CHRONIC ESSENTIAL FATTY ACID DEFICIENCY IN MICE [1]

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FIVE FIGURES

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Since the discovery by Burr and Burr ('29) that the total absence of fat from the diet of rats resulted in a deficiency disease, considerable data have been accumulated on the subject. In the great majority of cases, the disease has been produced by placing weanling rats on a fat-free diet. The main external symptoms considered specific for the syndrome were the cessation of normal weight gain and various skin disorders, including scaliness of the feet and tail, dermatitis and dandruff. Kidney lesions and fatty livers were noticed on autopsy. However, these symptoms apparently did not occur in mature rats when placed on the diet after weaning. Barki and co-workers ('47) were able to produce essential fatty acid (EFA) deficiency symptoms in the mature rat only by maintaining the animals on a restricted diet until they had lost one-half their weight and then placing them on a fat-free diet fed ad libitum.

It is the purpose of this paper to show that in the case of the mature animal, even though no symptoms may appear, a fat-free diet brings about a chronic deficiency condition which will not permit the animal to meet a metabolic stress

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successfully. In other words, what was shown years ago to hold true for vitamins may now be said to be true for the essential fatty acids.

Mice were used in these experiments even though only one study of essential fatty acid deficiency using these animals has been reported (White, Foy and Cerecedo, '43). Mice were thought to have several advantages. Greater numbers could be used without necessitating the preparation of excessive amounts of diet. The symptoms shown in acute deficiency by mice are more easily recognized than those exhibited by rats and occur more quickly. Finally, mice were being used in a radiation experiment (see below), the results of which could be compared with those of the fat-deficiency experiments.

EXPERIMENTAL

Materials and equipment

Cages and powdered food feeders [2] were designed and constructed at this Project. A commercial stock diet for rats was employed.[3] The sources of the essential constituents of the other diets are listed in the footnotes to table 1.[4]

Methods

An artificial diet, supplied to the various test groups, was compounded as shown in table 1. The fat-soluble vitamin supplements were dissolved in ethanol solution according to the following composition (per 100 ml): β-carotene 49 mg, calciferol 50 mg, α-tocopherol 300 mg. One milliliter of this mixture was thoroughly incorporated into 150 gm of the artificial diet on Monday, Wednesday and Friday of each week.

[2] These containers were designed by us to enable the mice to get the powdered food with ease, without scattering it about the cage. They consist of 8-oz. opaque ointment jars. The screw caps of these jars are provided with 7 small holes (usually five-eighths inch for average size mice) and one large hole holding an inverted wide-mouth bottle which serves as a constant leveling device.

[3] Rockland rat diet, obtained from the Rockland Farms.

[4] The albino mice used were in part supplied by the California Caviary and in part from our animal colony.

Corn oil [5] was supplied ad libitum to one of the test groups. This oil contains over 60% of linoleic acid.

The feeding of all animals was standardized in the following manner. At 10:00 o'clock each morning the food remaining in the containers was weighed and discarded and an amount of fresh food was supplied to cover the needs for 24 hours plus a 100% excess. The fat-soluble vitamins in alcohol solution were thoroughly mixed in the diet three times a week (on Mondays, Wednesdays and Fridays). The water-

TABLE 1

Composition of experimental diets

INGREDIENT	RATION		VITAMIN SUPPLEMENT PER KG OF DIET [4]	
	Fat free	Fat supp.		
	%	%		gm
Casein, vitamin-free [1]	20	20	Ascorbic acid	0.458
Sucrose [2]	72	72	Biotin	0.0000045
Salt mixture 2 [1]	4	4	Calcium pantothenate	0.0642
Cellu flour [3]	4	4	Choline chloride	1.2
Mazola corn oil [2]	..	Ad libitum	Folic acid	0.113
			Inositol	1.2
			2-Methyl-1,4-naphthoquinone	0.006
			Niacinamide	0.0605
			Para-aminobenzoic acid	0.60
			Pyridoxine hydrochloride	0.029
			Riboflavin	0.0274
			Thiamine hydrochloride	0.071
			β-Carotene	0.0049
			Calciferol	0.005
			α-Tocopherol acetate	0.030

[1] Purchased from the Nutritional Biochemicals Company.

[2] Purchased from a local grocery.

[3] Purchased from the Chicago Dietetic Supply Co.

[4] The ascorbic acid, calcium pantothenate, 2-methyl-1,4-naphthoquinone, niacinamide, pyridoxine hydrochloride, riboflavin and α-tocopherol acetate were purchased from Merck and Co.; the biotin, choline chloride, folic acid, inositol, para-aminobenzoic acid, thiamine hydrochloride and β-carotene were purchased from the Nutritional Biochemicals Co.; the calciferol was purchased from the Winthrop Chemical Company.

[5] Mazola.

soluble vitamins were thoroughly incorporated in the diet at the time of mixing.

Corn oil was supplied in separate containers similar in construction to the feeders, except that smaller ointment jars were used and upright bottles were not thought necessary. These containers were weighed daily and the average consumption of corn oil was determined. Throughout the experiments, the daily average per mouse per day was 35 mg.

Lettuce, purchased from a local grocery and thoroughly washed before being placed in the cages, was supplied twice weekly to our control animals on the commercial stock diet.

Cages were cleaned once a week in order to prevent the animals from obtaining fat by coprophagy.

Periodical checks were made to ascertain whether the animals were infested with pathogenic parasites, both internal and external. Diseased animals were removed from the test groups (only two cases of labyrinthitis occurred during the entire experimental period). Post mortem examinations were made as soon as possible after death.

RESULTS

A. Production of the acute type of essential fatty acid deficiency

As a check on the diet, the results of White, Foy and Cerecedo ('43) have been confirmed. In a typical experiment the growth of weanling mice of a strain reared in this laboratory and averaging 11 gm when placed on the diet, was studied. Figure 1 shows the rate of growth of the fat deficiency animals as compared with that of the controls on the commercial stock diet. There are several points of interest in the curves. The initial rapid gain in weight of the animals on the fat-free diet as compared with those on the normal diet precedes in every experiment the rapid weight gain of the controls, which soon far surpass the experimental animals in this respect. The weight plateau reached by all experimental animals is below that for the control females

and far below that for the control males. The fat-deficient males, however, were smaller than the corresponding females and succumbed to the effects of the diet in every case; whereas the females, though in poor condition, had all survived after 4 months on the diet. Finally, the resumption

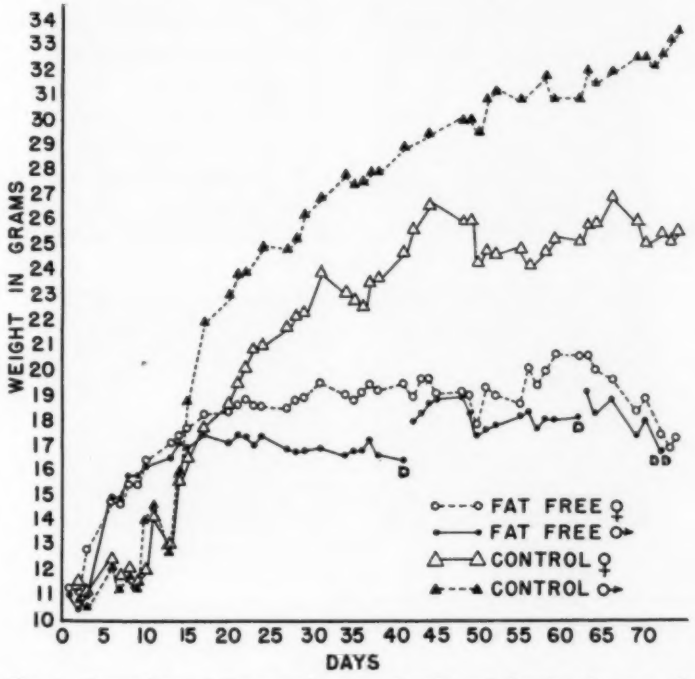

Fig. 1 Comparison of the growth rates of male and female mice on a fat-free diet from weaning with that of controls on a commercial stock diet. The death of an animal is indicated by a "D" followed by a discontinuity.

of weight gain by survivors when placed on the commercial stock diet or when given a corn oil supplement can be seen in figure 2. The preliminary rate of growth of those placed on the commercial diet may be due to its greater palatability to the mice, or to the lower weights of these mice when placed on this diet.

The symptoms shown by the acute EFA-deficient mice used in our experiments generally agreed with those described by White, Foy and Cerecedo and included loss of hair, dandruff, dermatitis (including a type we have termed "rope of sand dermatitis," because of its resemblance to a thin line of sand

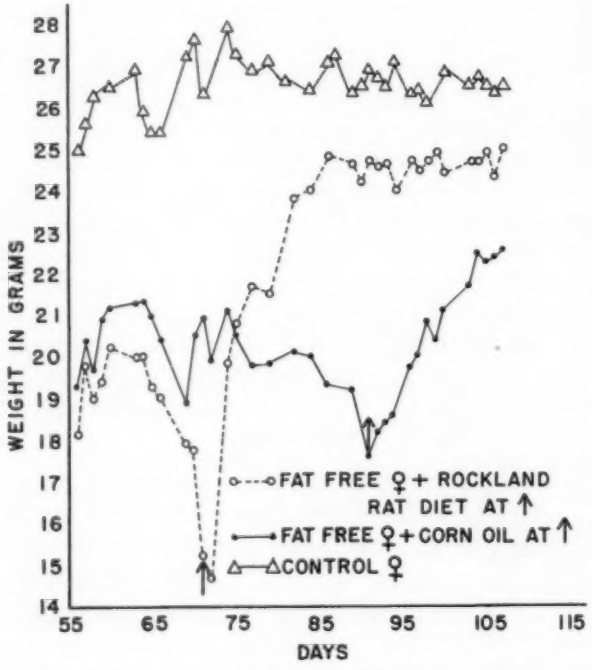

Fig. 2 The recovery of the growth rate of EFA-deficient female mice when placed on a corn oil-supplemented diet or on a commercial stock diet at the points indicated.

sprinkled along the skin between the fore and hind leg), diarrhea (at times bloody) and scaliness and crustiness of the skin. Post mortem examinations, carried out immediately after the death of the animal where possible, showed the following symptoms: the skin was very dry, and a lack of

peritoneal fluid was evident; the liver, kidneys, lungs and spleen appeared normal, but were not examined microscopically. The external appearances of some typical experimental animals are shown in figure 3.

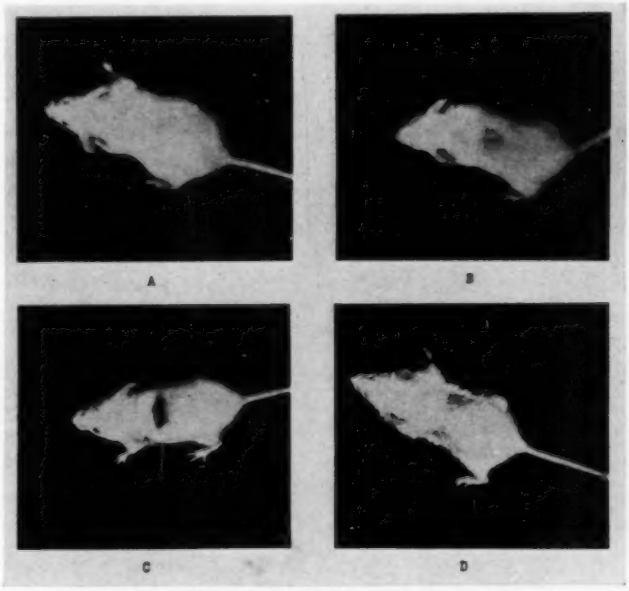

Fig. 3 Typical mice from an acute EFA deficiency experiment; A, commercial diet controls; B, C, D, acute EFA-deficient animals showing loss of hair, unkempt appearance and small size.

B. Production of the chronic type of essential fatty acid deficiency

In general, the methods used for the production of the chronically deficient mice were the same as those described above, except that the mice were placed on the fat-free diet at an average weight of 20 gm. These animals gained weight almost as rapidly as the controls and did not show any external symptoms of EFA deficiency. Superficially, they ap-

peared to be be perfectly normal, healthy animals, even after 8 months. However, their water consumption was considerably above normal (as is the case with the acutely deficient animals), and upon dissection every animal displayed a fatty

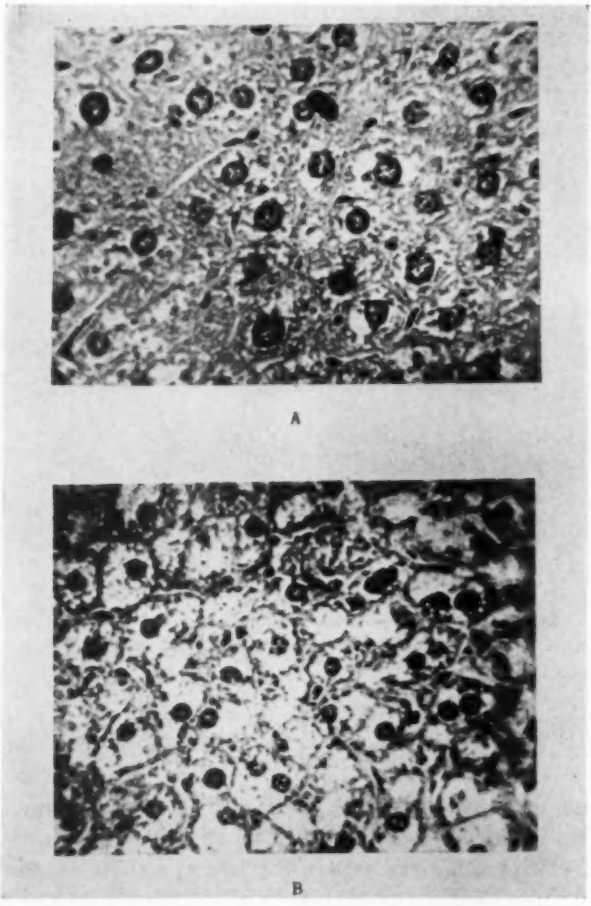

Fig. 4 Photomicrographs of liver tissue from: A, a commercial stock diet control mouse; B, a chronically EFA-deficient mouse. × 800.

liver (fig. 4). That these animals were indeed in a pathologic state has been demonstrated in several experiments.

I. The effect of injury upon the chronic essential fatty acid-deficient mouse. This experiment was initiated in order to follow the course of the deficiency disease, if possible, by blood counts taken at regular intervals.[6] Three groups of mice were used in this experiment — 10 controls on the commercial stock diet, 10 controls on a fat-free diet plus corn oil, and 30 experimental animals on the fat-free diet. Blood counts and smears were made in the usual manner for peripheral blood, being obtained from a small cut in the tail.[7] In the case of the control groups, the wounds healed rapidly and the animals showed no ill effects from the experience. As can be seen from figure 5, no significant difference in weight gain existed between those control animals which had their blood counts taken and those which did not. In the case of the chronically fat-deficient animals, however, the picture is quite different. In this case the wounds did not heal and the tails usually became necrotic and were lost. The animals lost weight, began to show typical symptoms of the acute type of fat deficiency, and finally died. These results are also shown in figure 5, in which the deaths are recorded as well as the weight changes (all those animals which had their blood counts taken died). An important point to keep in mind in this connection is that the death of one or more animals usually results in a weight increase in the remainder, since those which die are generally the smallest.

Finally, it can clearly be seen that the chronically fat-deficient animals were unable to survive a stress which had little or no effect on those on a normal diet. Whether the loss of blood (however slight) caused by the wounds contributed significantly to the results has not been ascertained.

One further indication of the use of essential fatty acids in injury may be noted. An accidental injury occurred in

[6] A correlation between essential fatty acid deficiency and white cell count was suspected but has not as yet been confirmed.

[7] Performed by the Hematology Section of this Project.

one of the mice in the corn oil control group. It was immediately separated from the main group but received exactly the same treatment and was kept under the same conditions. During the course of healing, this animal consistently consumed from two to three times the daily amount of corn oil consumed by the other mice of the group. Admittedly, little conclusion can be drawn from this one example. However, when considered with the whole experiment it may serve as further evidence of a need for the essential fatty acids in the healing of wounds.

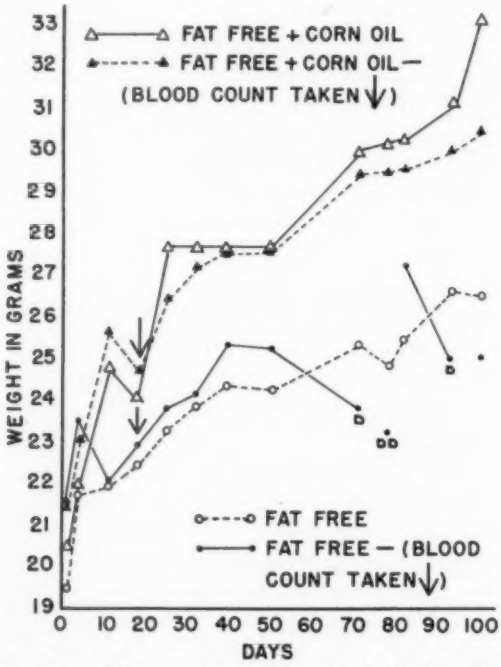

Fig. 5 The effect of injury on the growth rate of chronically EFA-deficient female mice as compared to that of controls fed a corn oil supplement. The arrows indicate the date on which blood counts were taken. The death of an animal is indicated by a "D" followed by a discontinuity. The change of slope for all animals at about the 40th day represents an extreme of temperature.

II. The effect of chronic essential fatty acid deficiency upon fertility. In this experiment, 25 female mice were placed on the fat-free diet at an average weight of 20 gm. Their appearance and weight gain were typical of that noted for chronically fat-deficient mice in previous experiments. Vaginal smears were taken at regular intervals to ascertain the effects of the diet on the estrous cycle. Little effect was noted, as all the females appeared to have a fairly normal cycle. After 60 days on the diet, when an average weight of 23.5 gm had been attained, the males were introduced in the following manner. One male of proved virility was placed in each of the 5 cages of females and left for one week. At the end of this period the males were removed and were replaced by others. This process was repeated a total of 4 times. Signs of sexual activity were apparent in both males and females throughout the test period. During this period the females were checked carefully for weight changes and altered appearance. Some weight increases were noted, but in no case was any definite pregnancy found. Several of the animals developed fat deficiency symptoms resulting, in at least one case, in death. These results partially confirm but are not in complete agreement with those of Burr and Burr ('30), Evans, Lepkovsky and Murphy ('34) or Quackenbush, Kummerow and Steenbock ('42), all of whom, using female rats which been on a low-fat diet from weaning, reported that litters were produced in most cases even though all the young and some of the mothers died.

It is possible, in the case of our experiments, that the weight increases and the appearance of fat deficiency symptoms were the result of temporary pregnancy. Autopsies which were performed on all animals 6 weeks after the introduction of the males were probably too late to confirm this, and indeed showed no definite signs of previous conception. The chronically fat-deficient female mouse is evidently completely incapable of producing young.

III. The effect of x-irradiation on the chronic fat-deficient mouse. In a typical experiment, 35 mice were divided into

the following groups. Group I (9 animals) were on the commercial stock diet. Group II (9 animals) were on a fat-free diet plus corn oil. Groups III (9 animals) and IV (8 animals) had been on a fat-free diet for 70 days. They showed little effect from the diet and were, to external appearances, normal.

All of the above groups were irradiated with a dosage of 500 r whole-body radiation, which is slightly greater than the mid-lethal dose for mice in this laboratory. Immediately after irradiation, the animals were replaced in their cages and the diet of group III (fat-free diet) was supplemented with corn oil, while the other groups were kept on their respective diets.

In group I, 5 of the 9 animals survived. Each of the animals in this group which did not survive developed a peculiar edema of the head, particularly on the cheeks, from about one to one and one-half days prior to death. No apparent reason can be given for this observation other than possible increased permeability of tissue caused by radiation or some type of infection which did not affect the other cases. Post mortem examinations made on these animals revealed a normal appearance of the spleen and kidneys, slight intestinal damage, some slight anemia of the liver lobes and some cases of lung hemorrhage. The survivors of this group were active and healthy in appearance, gained more weight with the passage of time, and did not develop any edema.

Eight survivors remained of the 9 group II corn oil controls. Post mortem examinations of the one mouse which died revealed a pathological picture similar to that described in connection with group I, but without edema.

No survivors remained of the 9 group III animals on the fat-free diet with corn oil added after radiation. Gross pathological examinations showed a gelatinous intestinal degeneration which was more severe than in any of the other groups examined. Bloody diarrhea was common among the animals in this group and autopsy confirmed this. The liver lobes were very anemic but the spleen and kidneys appeared

normal, although paler in color. The lungs were hemorrhagic in several of the mice examined. In general, those animals were emaciated, anorexic, listless and irritable. Most had lost some hair and some exhibited the typical dermatitis of the acute type of EFA deficiency.

In group IV, 2 of the 8 animals survived. The behavior and pathological appearance of this group were similar to those of group II except that bloody diarrhea was not as common and the intestines of the animals examined did not exhibit as great a degeneration.

DISCUSSION

On the basis of the results reported above, it is clear that an animal may be brought to an EFA-deficient state even though placed on the fat-free diet well after weaning. In this so-called chronic state of deficiency none of the external symptoms charactistic of the acute state may be apparent, and the animal may grow normally and appear to be in good condition. However, in such a state he may not be able to meet adequately any stress requiring rapid growth or new cell formation. Stresses of this type, as reported above, may be produced by pregnancy, radiation damage and traumas of other sorts. Under these conditions, the chronically essential-fatty-acid-deficient animal rapidly develops the symptoms of the acute state and, depending on the extent of the injury, may quickly succumb.

In the case of the chronic EFA-deficient mouse, therefore, we have an animal which, because of the efficiency with which the essential fatty acids are selected from the dietary fat and the tenacity with which they are kept from entering the metabolic pool, is in a static state. Essential fatty acids are neither received nor destroyed at an appreciable rate.[8] How-

[8] Mazza and Marfori ('41) claim that the essential fatty acids are not dehydrogenated by their enzyme systems derived from rat liver; while Rieckehoff, Holman and Burr ('49) and Barki, Collins, Hart and Elvehjem ('49) have shown that the tissue fatty acids of the rat on a fat-free diet retain considerable amounts of unsaturated fatty acids.

ever, if a metabolic stress is produced requiring the use of the stored essential fatty acids in the rapid formation of new cells (Smedley-MacLean and Hume, '41), large enough quantities may be used so that the remainder is inadequate for the carrying out of normal body functions.

The exact mechanism of the action of the essential fatty acids is still obscure, although it is becoming increasingly apparent that it must be intimately associated with the formation of new cells. It is possible that this function may be basic to a good many of the conditions noted in EFA deficiency, since the skin, for example, is an organ requiring the constant formation of new tissue. However, further speculation must await the completion of studies now in progress in this laboratory designed specifically to elucidate the mechanism of action of these compounds.

Any inferences resulting from these experiments and applied to the case of the human being would be tenuous, although experiments are now in progress in this laboratory which are designed to test the applicability of our results to larger animals. However, as Burr ('42) and others have pointed out, the present trend in modern civilized communities toward diets high in purified carbohydrate could very well lead to a state in which insufficient essential fatty acids are received. The above experiments show that such a state might not become apparent under normal conditions, but that in the event of any metabolic stress it would be hazardous indeed.

SUMMARY

It has been shown that a state of chronic essential fatty acid deficiency may be produced in mature mice. In this state the external appearance is normal, but typical acute EFA deficiency symptoms, and possibly death, may be produced by the following stresses:

(1) Injuries (even minor); (2) pregnancy (the chronic EFA-deficient females are sterile); (3) x-irradiation.

LITERATURE CITED

BARKI, V. H., R. A. COLLINS, E. B. HART AND C. A. ELVEHJEM 1949 Relation of fat deficiency symptoms to the polyunsaturated fatty acid content of the tissues of the mature rat. Proc. Soc. Exp. Biol. Med., *71:* 694–696.

BARKI, V., H. NATH, E. HART AND C. A. ELVEHJEM 1947 Production of essential fat acid deficiency symptoms in the mature rat. Ibid., *66:* 474–478.

BURR, G. O. 1942 Significance of the essential fat acids. Fed. Proc., *1:* 224–233.

BURR, G. O., AND M. M. BURR 1929 A new deficiency disease produced by the rigid exclusion of fat from the diet. J. Biol. Chem., *82:* 345–367.

———— 1930 On the nature and role of the fatty acids essential in nutrition. Ibid., *86:* 587.

EVANS, H. M., S. LEPKOVSKY AND E. A. MURPHY 1934 Vital need of the body for certain unsaturated fatty acids. Ibid., *106:* 431.

MAZZA, F. P., AND L. MARFORI 1941 La deidrogenasi degli acidi grassi superiori. Arch sci. biol. (Italy), *27:* 142–162.

QUACKENBUSH, F. W., F. A. KUMMEROW AND H. STEENBOCK 1942 The effectiveness of linoleic, arachidonic and linolenic acids in reproduction and lactation. J. Nutrition, *24:* 213–224.

RIECKEHOFF, I. G., R. T. HOLMAN AND G. O. BURR 1949 Polyethenoid fatty acid metabolism — effect of dietary fat on polyethenoid fatty acids of rat tissues. Arch. Biochem., *20:* 331–340.

SMEDLEY-MACLEAN, I., AND E. HUME 1941 Fat deficiency disease of rat — the storage of fat in the fat starved rat. Biochem. J., *35:* 990–995.

WHITE, E. A., J. R. FOY AND L. CERECEDO 1943 Essential fat acid deficiency in the mouse. Proc. Soc. Exp. Biol. Med., *24:* 301–302.

URINARY AND FECAL ELIMINATION OF B_6 AND 4-PYRIDOXIC ACID ON THREE LEVELS OF INTAKE[1]

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The synthesis of vitamin B_6 has been demonstrated in the rumen of the cow (Wegner et al., '40; McElroy and Goss, '40), in the rumen of the sheep (McElroy and Goss, '39), in the cecum of the rat (Mitchell and Isbell, '42), and in the intestinal tract of the horse (Carroll et al., '49). Studies of vitamin B_6 metabolism in human subjects have been reported by Denko et al. ('46), Johnson et al. ('45), Huff and Perlzweig ('44), and Rabinowitz and Snell ('49). However, the total vitamin B_6 intake and the total elimination of vitamin B_6 and metabolites were not reported by any of these investigators.

The present study deals with the results of a complete balance study of vitamin B_6 and its known metabolites on human subjects on three levels of vitamin B_6 intake, and of the effect of sulfasuxidine (succinylsulfathiazole) on the total output at the lowest level of vitamin intake.

EXPERIMENTAL

The same basal diet was used for the 4 experimental periods of the study. In the first period it was fed without supplement; in the other three periods it was supplemented

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daily as follows: (a) by 2 mg pyridoxine HCl, (b) by 15 mg pyridoxine HCl, or (c) by 12 gm of sulfasuxidine.

Dietary periods were preceded by a 4-day depletion diet, with the exception of the sulfasuxidine period. Details of the dietary periods are included in the tables.

The depletion diet consisted of fruit, vegetables and rice and furnished approximately 7 gm protein and 780 cal. per day. Additional calories in the form of butter and sugar were given to meet the energy requirement. This depletion diet was introduced primarily for the purposes of another study.

The basal diet was also an all-vegetable diet. It differed from the depletion diet through the addition of oatmeal cookies, cornmeal and potatoes. The food intake for each subject remained constant throughout the experimental period, but the amount of these foods varied among subjects according to their size. Consequently, the protein content varied from 20 to 30 gm and the vitamin B_6 from 0.53 to 1.21 mg daily for different individuals. Fat and sugar were added to bring the total caloric intake to 40 cal. per kilogram body weight. Daily supplements of calcium lactate (600 mg), thiamine HCl (2 mg), riboflavin (3 mg) and niacin amide (20 mg) were given. Vitamins A and C occurred in the food in sufficient quantities to meet the N.R.C. Recommended Dietary Allowances. Carbohydrate supplied approximately 46%, fat 50% and protein 4% of total caloric intake. Most of the food used for one experiment was purchased at one time to eliminate variation as much as possible. Daily aliquots of food were saved and analyzed to give the values for the vitamin B_6 content of the basal diet.

The subjects were 10 young women and three young men ranging in age from 22 to 30 years. All were graduate students or the wives of graduate students. They were considered to be in good health and carried on their usual activities throughout the experiment.

Twenty-four-hour urine collections were made in brown bottles with toluol as a preservative. Fecal collections were

made in three-day periods, using carmine as a marker. These were preserved in acid-alcohol. The microbiological method of Atkin et al. ('43) was used for the determination of vitamin B_6. The content of 4-pyridoxic acid was determined by the fluorometric method of Huff and Perlzweig ('44). These workers found 4-pyridoxic acid to be the main metabolite excreted after ingestion of pyridoxine, and established its identity. It has been shown by Sarett and Goldsmith ('49) that fluorescent compounds present in the urine following the administration of large quantities of tryptophan are responsible for increasing the apparent values for 4-pyridoxic acid. These interfering substances are removed by extraction with ether. Although the diet used in this investigation was very low in protein, and consequently low in tryptophan, ether extractions of the urine were made. The 4-pyridoxic acid values of the ether-extracted urine did not differ from those of the urine before extraction with ether.

RESULTS AND DISCUSSION

Data on vitamin B_6 intake and on the elimination of urinary vitamin B_6 and 4-pyridoxic acid and fecal vitamin B_6 are presented in tables 1, 2 and 3. These values represent the individual averages for each period.

Basal diet

Examination of the data in these tables shows that the total elimination of vitamin B_6 and 4-pyridoxic acid by subjects on the basal diet was considerably greater than the vitamin intake. The dietary intake of vitamin B_6 averaged 0.78 mg daily for the 16 subjects. The average total elimination of urinary vitamin B_6 and 4-pyridoxic acid and fecal vitamin B_6 was 3.54 mg, or 2.76 mg above intake. The range of total elimination over intake was 1.63 to 4.71 mg per day. The main excretory product was 4-pyridoxic acid, which comprised 75% of the total elimination and 96% of the urinary excretion.

TABLE 1

Elimination of vitamin B_6 and 4-pyridoxic acid on three levels of intake
(Vitamin B_6 expressed as mg pyridoxine HCl per day)

SUBJECT	VITAMIN B_6 CONTENT OF BASAL DIET	ELIMINATION OF VITAMIN B_6 AND 4-PYRIDOXIC ACID (AVERAGE PER DAY)											
		Basal diet [1]				Basal plus 15 mg pyridoxine HCl				Basal plus 2 mg pyridoxine HCl			
		Vitamin B_6		Urinary 4-pyridoxic acid	Total	Vitamin B_6		Urinary 4-pyridoxic acid	Total	Vitamin B_6		Urinary 4-pyridoxic acid	Total
		feces	urine			feces	urine			feces	urine		
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
C.B.	1.21	1.13	0.12	2.52	3.77	1.21	0.64	13.38	15.33	1.19	0.14	4.03	5.36
P.S.	0.89	0.72	0.12	2.59	3.43	0.79	0.66	7.84	9.29	0.85	0.14	3.16	4.15
A.R.	0.65	0.57	0.06	1.65	2.28	0.80	0.61	12.35	13.76	0.73	0.13	3.43	4.29
J.P.	0.97	0.81	0.08	2.18	3.07	0.48	0.83	7.92	9.23				
E.P.	0.97	0.84	0.10	2.18	3.12	0.49	1.09	7.23	8.81				
H.L.	0.53	0.61	0.08	2.68	3.37	0.88	1.15	8.15	10.18				
L.O.	0.53	0.58	0.11	2.13	2.82	0.82	0.95	6.73	8.50				
Ju.P.	0.53	0.55	0.10	2.82	3.47	0.84	1.75	7.97	10.56				
Ge.P.	0.53	0.55	0.08	2.69	3.32	0.95	1.29	8.29	10.53				
Ave.	0.76	0.71	0.09	2.38	3.18	0.81	1.00	8.87	10.69	0.92	0.14	3.54	4.60

[1] An interval of at least two weeks on a normal diet intervened between the above periods. Each experimental period was preceded by the 4-day depletion diet. The experimental periods were 10 days in length. Complete urinary and fecal collections were made for the last 9 days.

TABLE 2

Effect of 15-mg supplement of pyridoxine HCl on elimination in subsequent periods [1]

SUBJECT	VITAMIN B_6 CONTENT OF BASAL DIET	ELIMINATION OF VITAMIN B_6 AND 4-PYRIDOXIC ACID (AVERAGE PER DAY)											
		Basal diet				Basal diet plus 15 mg pyridoxine HCl				Basal diet			
		As vitamin B_6		Urinary 4-pyridoxic acid	Total	As vitamin B_6		Urinary 4-pyridoxic acid	Total	As vitamin B_6		Urinary 4-pyridoxic acid	Total
		feces	urine			feces	urine			feces	urine		
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
P.S.	0.65	0.61	0.12	2.64	3.37	0.76	0.53	10.32	11.61	0.81	0.20	5.25	6.26
O.W.	0.58	0.51	0.09	2.06	2.66	0.70	0.69	7.12	8.51	1.20	0.29	3.85	5.34
A.R.	0.53	0.56	0.06	1.95	2.57	0.65	0.52	7.46	8.63	0.63	0.09	3.85	4.57
Ave.	0.58	0.56	0.09	2.22	2.87	0.70	0.58	8.30	9.58	0.88	0.19	4.32	5.39

[1] A 4-day depletion diet preceded this study.

TABLE 3

Effect of 12 gm sulfasuxidine per day upon the elimination of vitamin B_6 and 4-pyridoxic acid by human subjects on a low vitamin B_6 intake (expressed as mg pyridoxine HCl per day) [1]

SUBJECT	VITAMIN B_6 CONTENT OF BASAL DIET	VITAMIN B_6 AND 4-PYRIDOXIC ACID ELIMINATION (AVERAGE PER DAY)							
		Basal diet				Basal plus sulfasuxidine			
		As vitamin B_6		Urinary 4-pyridoxic acid	Total	As vitamin B_6		Urinary 4-pyridoxic acid	Total
		feces	urine			feces	urine		
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
H.L.	0.97	1.12	0.26	4.30	5.68	1.32	0.20	4.25	5.77
J.Z.	0.97	0.64	0.22	4.40	5.26	1.37	0.25	4.40	6.02
E.K.	0.97	0.55	0.16	3.25	3.96	0.79	0.15	3.50	4.44
J.R.	0.97	0.70	0.22	3.60	4.52	0.80	0.18	3.60	4.58
Ave.	0.97	0.75	0.21	3.89	4.85	1.07	0.20	3.94	5.20

[1] This study was not preceded by the depletion diet. Periods were 6 days in length.

The total "vitamin B_6" eliminated via the urine and feces approximated or was slightly in excess of the amount in the diet. Approximately 85% appeared in the feces and 15% in the urine. These results differ from those of Denko et al. ('46), who found a total elimination of 0.52 mg of vitamin B_6 daily from subjects on an intake of 1.05 mg. The higher elimination values in our study are due to the consistently higher fecal output of our subjects.

Two possible explanations for the increase in elimination of B_6 and metabolites over intake occur to us. One is that the excess represents a lag in excretion following a higher dietary intake; the other, that synthesis has taken place. Data on three successive three-day periods support the latter view, inasmuch as the trend for the 9 days shows an increase rather than a decrease in elimination as the period progresses. (Average total elimination per day for three successive periods was 2.59, 3.24 and 3.60 mg.) None of the 12 subjects showed a decrease in elimination.

Basal diet plus 2 mg pyridoxine HCl

Study of the data in table 1 on the three subjects given a 2-mg supplement of pyridoxine HCl shows that the total elimination exceeded the intake by an average of 1.68 mg per day. The 2-mg supplement increased only slightly the output of vitamin B_6 over that eliminated on the basal diet. The main increase occurred in the excretion of 4-pyridoxic acid, which constituted 77% of the total elimination and 95% of the urinary excretion.

Basal diet plus 15 mg pyridoxine HCl

When a 15-mg supplement of pyridoxine HCl was given daily, the total elimination of vitamin B_6 and metabolites was found to be 67% of the intake (table 1). In this test 4-pyridoxic acid constituted 83% of the total elimination and 88% of the urinary excretion. The urinary excretion of the vitamin, although still representing a small percentage of

the total elimination, was approximately 10 times the amount excreted as the vitamin on the basal diet.

Little or no increase in fecal vitamin B_6 took place after the ingestion of 15 mg of pyridoxine HCl, indicating almost complete absorption or a masking of any failure in absorption through destruction by microorganisms. Denko et al. ('46) also reported that fecal elimination was unaffected by oral vitamin supplementation.

Failure to obtain higher recoveries of vitamin B_6 and 4-pyridoxic acid after 15-mg supplements of pyridoxine HCl may be due to the presence of unidentified excretion products or to complete oxidation within the body of part of the vitamin, as has been suggested by Rabinowitz and Snell ('49).

Effect of 15-mg supplement of pyridoxine HCl on elimination of vitamin B_6 metabolites in subsequent periods

In this study a 9-day period of supplementation by 15 mg pyridoxine was preceded by 9 days on the basal diet and followed by 6 days on the basal diet. That a part of the vitamin given during the supplemented period is stored in the body is indicated by the lag in excretion shown in table 2. After 6 days on the restricted diet following the 9-day 15-mg supplementation period excretion had not yet returned to the level on the basal diet in the earlier period, although there had been a steady day-to-day decline in 4-pyridoxic acid excretion. Rabinowitz and Snell ('49), in a study of subjects on a normal diet, reported that excretion returns to normal levels within 12 hours after a single ingestion of a 100-mg dose. The 4-day depletion diet preceding the 24-day experimental period may have caused tissue depletion in our subjects. The vitamin B_6 content of the depletion diet was not determined, but it, without doubt, would be considerably lower than that of the basal diet. It is also possible that the low starch content of the depletion diet depressed intestinal synthesis during this period. Further evidence that the depletion diet resulted in loss of vitamin B_6 from

the body is indicated by a comparison of the excretion data of the subjects on the basal diet shown in tables 1, 2 and 3. Subjects represented in tables 1 and 2 were on the depletion diet, while those represented in table 3 were not. Both urinary vitamin B_6 and urinary 4-pyridoxic acid were greater in the subjects reported in table 3.

Basal diet plus 12 gm sulfasuxidine

The administration of sulfasuxidine for a period of 6 days following a 6-day period on the basal diet resulted in no decrease in output of vitamin B_6 and 4-pyridoxic acid (table 3). Actually the total elimination was slightly higher when sulfasuxidine was taken. The increase was found in the feces. The urinary excretion of the vitamin and of 4-pyridoxic acid was almost identical in both periods. The increased fecal elimination may have been due to a change in the type of intestinal flora, resulting in fewer of the organisms which may normally use or destroy the vitamin in the intestinal tract. Similar increases in fecal riboflavin in man (Najjar et al., '44), and in riboflavin and pantothenic acid in rabbits (Olcese et al., '48), have been noted during ingestion of sulfasuxidine. The possibility remains either that synthesis of vitamin B_6 takes place outside the digestive tract, and that destruction of bacteria in the tract would, therefore, have no effect on depressing synthesis, or that the destruction of bacteria takes place largely in the colon after the synthesized vitamin has been absorbed.

Evidence that the values found for 4-pyridoxic acid are actually a measure of the oxidation product of vitamin B_6 is shown by further study of the data. It has already been mentioned that on the basal diet 96% of the total urinary excretion of vitamin B_6 and 4-pyridoxic acid was present as the latter. The urinary recovery of the supplemented vitamin may be calculated by subtracting urinary vitamin B_6 and 4-pyridoxic acid excreted by subjects on the basal diet from that excreted by subjects on the basal diet plus vitamin supplementation. The urinary recovery of a 2-mg supplement

of pyridoxine HCl was 1.33 mg. Of this, 97% occurred as 4-pyridoxic acid. This seems sufficient evidence that few if any fluorescent substances interfered with the determination of 4-pyridoxic acid in this study. The amount recovered as 4-pyridoxic acid on the 15-mg supplement was 87%. It has been reported that 70% of the recovery of a 100-mg supplement occurred as 4-pyridoxic acid (Rabinowitz and Snell, '49). The percentage excreted as the vitamin on these higher intakes may possibly be explained by a rapid removal of the vitamin from the blood by the kidney before oxidation has had time to take place. The fact that the major portion of the metabolites excreted by our subjects on the basal, unsupplemented diet occurred as urinary 4-pyridoxic acid gives evidence of the absorption and utilization of vitamin B_6 synthesized in the tract. It would seem then that the vitamin B_6 synthesized in the human digestive tract is absorbed as rapidly and completely as that given orally. The apparent synthesis of vitamin B_6 found in this investigation may explain the difficulty encountered by Hawkins and Barsky ('48) in demonstrating symptoms of vitamin B_6 deficiency in man.

SUMMARY

Evidence of the synthesis of vitamin B_6 in man has been presented. Elimination of fecal vitamin B_6 and urinary vitamin B_6 and 4-pyridoxic acid on a dietary intake of 0.78 mg was 3.54 mg. The 4-pyridoxic acid accounted for 75% of the total elimination. Data are given which show that the 4-pyridoxic acid values are a measure of the oxidation product of vitamin B_6.

Vitamin B_6 alone eliminated in the feces and urine approximated or was slightly in excess of the amount in the basal diet.

Daily elimination of vitamin B_6 and 4-pyridoxic acid on the basal diet supplemented by 2 mg pyridoxine HCl was found to be greater than the vitamin intake by 1.68 mg. Approximately 67% of the vitamin intake was recovered in the urine and feces when the basal diet was supplemented by

15 mg pyridoxine HCl. The daily addition of 12 gm sulfasuxidine to the basal diet failed to result in any decrease in the total elimination of vitamin B_6 and 4-pyridoxic acid.

LITERATURE CITED

ATKIN, L., A. S. SHULTZ, W. L. WILLIAMS AND C. M. FREY 1943 Yeast microbiological methods for determination of vitamins. Ind. Eng. Chem., Anal. Ed., *15:* 141.

CARROLL, F. D., HAROLD GOSS AND C. E. HOWELL 1949 The synthesis of B vitamins in the horse. J. Animal Sci., *8:* 290.

DENKO, C. W., W. E. GRUNDY, N. C. WHEELER, C. R. HENDERSON AND G. H. BERRYMAN 1946 The excretion of B-complex vitamins by normal adults on a restricted intake. Arch. Biochem., *11:* 109.

HAWKINS, W. W., AND JAMES BARSKY 1948 An experiment on human vitamin B_6 deprivation. Science, *108:* 284.

HUFF, J. W., AND W. A. PERLZWEIG 1944 A product of oxidative metabolism of pyridoxine, 2-methyl — 3-hydroxy — 4-carboxy — 5-hydroxymethyl — pyridine (4-pyridoxic acid). Isolation from urine, structure, and synthesis. J. Biol. Chem., *155:* 345.

JOHNSON, B. C., T. S. HAMILTON AND H. H. MITCHELL 1945 The excretion of pyridoxine, "pseudopyridoxine" and 4-pyridoxic acid in the urine and sweat of normal individuals. Ibid., *158:* 619.

MCELROY, L. W., AND H. GOSS 1939 Report on 4 members of the vitamin B complex synthesized in the rumen of the sheep. Ibid., *130:* 437.

———— 1940 A quantitative study of vitamins in the rumen contents of sheep and cows fed vitamin-low diets. II. Vitamin B_6 (pyridoxine). J. Nutrition, *20:* 541.

MITCHELL, H. K., AND E. R. ISBELL 1942 Intestinal bacterial synthesis as a source of B vitamins for the rat. Univ. Texas Publ. 4237, p. 125.

NAJJAR, V. A., G. A. JOHNS, G. C. MEDAIRY, G. FLEISCHMANN AND L. E. HOLT, JR. 1944 The biosynthesis of riboflavin in man. J. Am. Med. Assn., *126:* 357.

OLCESE, ORLANDO, P. B. PEARSON AND B. S. SCHWEIGERT 1948 The synthesis of certain B vitamins by the rabbit. J. Nutrition, *35:* 577.

RABINOWITZ, J. C., AND E. E. SNELL 1949 Vitamin B_6 group. XV. Urinary excretion of pyridoxal, pyridoxamine, pyridoxine, and 4-pyridoxic acid in human subjects. Proc. Soc. Exp. Biol. Med., *70:* 235.

SARETT, H. P., AND GRACE A. GOLDSMITH 1949 Tryptophan and nicotinic acid studies in man. J. Biol. Chem., *177:* 461.

SARMA, P. S., E. E. SNELL AND C. A. ELVEHJEM 1946 The vitamin B_6 group. VIII. Biological assays of pyridoxal, pyridoxamine and pyridoxine. Ibid., *165:* 55.

WEGNER, M. I., A. N. BOOTH, C. A. ELVEHJEM AND E. B. HART 1940 Rumen synthesis of the vitamin B complex. Proc. Soc. Exp. Biol. Med., *45:* 769.

THE EFFECT OF SUPPLEMENTATION OF HIGH CORN DIETS ON RAT REPRODUCTION AND LACTATION [1]

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INTRODUCTION

Extensive studies during recent years have repeatedly demonstrated that rations composed wholly or principally of corn and soybean meal supplemented with minerals and known vitamins are deficient with respect to normal reproduction and lactation of rats. Ross et al. ('42), Van Landingham and Lyon ('47), and Zucker and Zucker ('48) have all reported varying degrees of failure on such diets. Cary and Hartman ('43–'47) have found a partially purified ration containing highly extracted casein to be inadequate for normal reproduction. Recently these workers (Hartman et al., '49) have stated that the supplementation of these diets with vitamin B_{12} permits essentially normal performance. In contrast to these studies, Schultze ('50) has recently reported that essentially normal growth of young was obtained when rats were fed rations containing soybean protein supplemented with all the known vitamins except vitamin B_{12}. Ross et al. ('48) and Watts et al. ('49) have reported essentially normal reproduction of rats fed corn and soybean meal rations similarly supplemented. Gestational failure and high mortality of young were not consistently observed.

[1] Published with the approval of the Director, Oklahoma Agricultural Experiment Station.

EXPERIMENTAL

Assay techniques

Female rats of the Sprague-Dawley strain were used during the entire investigation. They were housed in large screen-bottom cages. Water and the various rations were supplied ad libitum. At time of breeding, males were placed with females and rotated daily to reduce the effect of possible male

TABLE 1

Composition of rations used in studies on reproduction of rats and growth of young to weaning

RATION COMPONENT	BASAL 2	BASAL 3	BASAL 4	BASAL 5
Ground yellow corn	68.85	73.0	83.0	53.0
Expeller soybean oil meal	25.00	25.0		45.0
Vitamin test casein			15.0	
Alfalfa leaf meal	5.00			
NaCl	0.65			
$CaCO_3$	0.50			
Salt mixture [1]		2.0	2.0	2.0
DL-Methionine		0.2	0.2	0.2
Vitamin mixture [2]		..	..	..

[1] Hegsted, J. Biol. Chem., *138:* 450 ('41).

[2] Basal rations were supplemented with a water soluble vitamin mixture which supplied in crystalline form the following amounts of the various vitamins per kilogram of rations: Thiamine HCl 4 mg, riboflavin 6 mg, pyridoxine HCl 3 mg, calcium pantothenate 20 mg, niacin 20 mg, choline chloride 1 gm, folic acid 1 mg, inositol 20 mg, *p*-aminobenzoic acid 20 mg. Two drops of a mixture of alpha-tocopherol and haliver oil were administered weekly.

sterility. At parturition the females were placed in individual cages provided with sugar cane bagasse as litter. Frequent observations permitted a fairly accurate assessment of the number born. Mortality data included both those born dead and those dying during the first 48 hours. After the first 48 hours, litters having more than 6 young were reduced to 6, females being selected preferentially. The young were weaned at 21 days of age. Weanling rats were selected for subsequent reproduction studies on the basis of weaning weight, litter, and ration. The young rats were fed various experimental

rations similar to those supplied the mother during gestation and lactation, and were bred at approximately 90 days of age, when they weighed about 200 gm. In some instances rats were maintained for three successive generations on the same ration.

The basal rations used in this study appear in table 1. Supplements when added were at the expense of the entire ration. The soybean meal [2] used during the entire study was a high quality expeller product. The casein was alcohol extracted.[3] The B_{12} supplement 1 [4] was a mixture of crude B_{12} concentrate, charcoal and soybean flour which, according to the manufacturer, had an activity roughly 4 times that of fish solubles. The B_{12} supplement 3 [5] was a Fuller's earth absorbate which, according to the manufacturer, had an activity of 12.5 mg of vitamin B_{12} per pound of the LLD *(Lactobacillus lactis* Dorner) assay. The APF (Animal Protein Factor) supplement [6] was a by-product of the manufacture of aureomycin. The antipernicious anemia liver extract solution [7] contained by manufacturer's assay 15 U.S.P. injectible units per milliliter.

Fractionation techniques

Repeated extraction of 1:20 liver powder [8] with 70% ethanol yielded two fractions, the 70% ethanol soluble fraction and the residue. The material soluble in 70% ethanol was concentrated *in vacuo* to a thick syrup and repeatedly extracted with 95% ethanol, yielding a soluble fraction and an insoluble fraction. Fractions of a high quality alfalfa leaf meal were prepared in essentially the same manner as liver. The 95% ethanol soluble and insoluble fractions of the material which was soluble in 70% alcohol were tested.

[2] Staley and Co., Decatur, Illinois.
[3] Product of General Biochemicals, Inc.
[4] Merck.
[5] See footnote 4.
[6] Lederle.
[7] See footnote 6.
[8] Wilson.

RESULTS AND DISCUSSION

In the early phases of this study various natural products and fractions were tested for activity in improving reproduction and lactation on corn-soybean meal rations. The results of one typical experiment are presented in table 2. There were no resorptions and toxemias observed, such as had been noted by Ross et al. ('42). The basal ration permitted the poorest performance during the first reproductive cycle, as regards the number of viable young at 48 hours, number of

TABLE 2

Effect of various fractions of 1:20 liver powder, APA [1] liver extract and other supplements on reproduction of rats and the growth of the young to weaning

EXPERIMENT	RATION	NUMBER OF LITTERS [2]	LIVABILITY AT 48 HRS.	YOUNG WEANED OF THOSE GIVEN TO RAISE	AVERAGE WEANING WEIGHT
			%	%	gm
	First reproductive cycle				
1	Basal 2	6	68	56	27.5
	Basal 2 + 2% 1:20 liver powder	7	97	85	36.5
	Basal 2 + ethanol insoluble fraction = 2%	7	96	83	36.6
	Basal 3 + ethanol soluble fraction = 2%	6	94	72	33.0
	Basal 2 + APA [1] liver extract = 0.4 ml/wk.	7	96	85	36.5
2	Basal 2 + 2% fish solubles	5	93	86	37.8
	Second reproductive cycle				
1A	Basal 2	5	88	78	34.9
	Basal 2 + 2% 1:20 liver powder	5	82	96	38.1
	Basal 2 + ethanol insoluble fraction = 2%	6	94	100	39.7
	Basal 2 + ethanol soluble fraction = 2%	2	100	100	37.0
	Basal 2 + APA [1] liver extract = 0.4 ml/wk.	4	88	91	37.8

[1] Antipernicious anemia liver extract.

[2] Number of litters denotes the number of females that actually produced litters and does not include those that failed to breed.

young weaned, and weaning weight. Various fractions of liver, APA (antipernicious anemia factor) liver extract [9] and fish solubles were all essentially equally active in supplementing the ration. Analysis of variance (Snedecor, '46) of the data showed that the differences in weaning weights observed were all highly significant when compared to those of the lot fed the basal ration. Although the females were maintained on the same ration for the second reproductive cycle, the differences were less striking. Statistical significance was not attained, although, in general, there was a tendency for the results to be in the same direction.

Since it has repeatedly been suggested that alfalfa contains a material which is beneficial for pregnant and lactating sows, studies were made with rats to test alfalfa leaf meal and fractions therefrom. Because basal ration 2 contained alfalfa, another ration (basal 3) was used in this study. This ration permitted normal gestation, good livability of young to 48 hours, and weaning of a high percentage of those given to raise (table 3). The addition of alfalfa leaf meal was definitely detrimental in terms of weaning weight. Fractions of alfalfa had no effect on weaning weight whereas, as previously observed, 1:20 liver powder gave a significant response.

The failure to produce high early mortality in the young rats in the above experiment made it seem desirable to use a ration compounded of materials of more definite composition. As a result basal ration 4 was formulated, using vitamin test casein as the source of protein. The results of two replications in which various supplements to this basal ration were tested appear in table 4. The animals used in experiment 6 were rats from the local stock colony maintained since weaning on the respective rations. The rats used in experiment 7 were young from experiment 6 fed from weaning the same rations fed their mothers. Again livability of the young to 48 hours was good in all lots and a high percentage of those

[9] See footnote 6, page 535.

given to raise was weaned. Liver powder [10] and B_{12} supplement 1 [11] significantly improved the weaning weights of the young on these rations over those of the lot fed the basal ration. The lack of reproductive failures in these two experiments may have been due to the presence of an unidentified factor in the incompletely extracted casein (Cary et al., '43–'47).

TABLE 3

Effect of various fractions of alfalfa leaf meal on reproduction of rats and the growth of the young to weaning

RATION	NUMBER OF LITTERS [1]	LIVABILITY AT 48 HRS.	YOUNG WEANED OF THOSE GIVEN TO RAISE	AVERAGE WEANING WEIGHT
		%	%	*gm*
Basal 3	6	94	80	37.0
Basal 3 + 15% alfalfa leaf meal	4	90	95	27.1
Basal 3 + 95% ethanol insoluble fraction of the 70% ethanol soluble fraction equivalent to 15% alfalfa leaf meal	4	91	82	39.4
Basal 3 + 95% ethanol soluble fraction of the 70% ethanol soluble fraction equivalent to 15% alfalfa leaf meal	3	97	89	38.1
Basal 3 + 2% 1:20 liver powder	6	100	100	42.9

[1] Number of litters denotes the number of females that actually produced litters and does not include those that failed to breed.

Since numerous workers have suggested that casein contains the unknown factor and that plant materials such as soybean meal are not active in this respect, further experiments were conducted using basal ration 3. Table 4 summarizes the results of the different experiments using this ration. The animals used in experiment 8 were the young from experiment 7 that had been maintained on the experi-

[10] See footnote 8, page 535.

[11] See footnote 4, page 535.

mental rations since weaning. The rats used in experiment 9 were young from experiment 8 and received the same ration as their mothers. The results of experiments 8 and 9 showed that no differences existed among the lots as to viability of

TABLE 4

Effect of various supplements on the reproduction of rats and the growth of the young to weaning

EXPERIMENT	RATION	NUMBER OF LITTERS [1]	LIVABILITY AT 48 HRS.	YOUNG WEANED OF THOSE GIVEN TO RAISE	AVERAGE WEANING WEIGHT
			%	%	gm
6,7	Basal 4	12	92	100	34.3
	Basal 4 + 0.5% Merck's B_{12} supplement 1	12	91	93	42.0
	Basal 4 + 2.0% Wilson's 1:20 liver powder	15	97	98	43.2
	Basal 4 + alfalfa carotene concentrate	8	96	96	38.0
8,9	Basal 3	15	95	95	38.5
	Basal 3 + 0.5% Merck's B_{12} supplement 3	14	93	99	41.0
	Basal 3 + 2.0% Wilson's 1:20 liver powder	18	95	97	40.4
10,11	Basal 3	11	68	91	37.3
	Basal 3 + 0.5% Merck's B_{12} supplement 3	10	71	97	40.4
12	Basal 3	7	85	94	37.0
	Basal 3 + 0.5% Lederle's APF supplement	6	88	100	44.0
	Basal 3 + 50 p.p.m. crystalline B_{12}	7	92	100	38.0
	Basal 3 + 0.5% Lederle's APF + 50 p.p.m. crystalline B_{12}	5	92	100	42.0
13,14	Basal 5	12	75	95	35.0
	Basal 5 + 0.5% Merck's B_{12} supplement 3	7	86	100	40.2

[1] Number of litters denotes the number of females that actually produced litters and does not include those that failed to breed.

the young up to 48 hours or the number weaned of those given to raise. As had been noted previously, the rats fed 1:20 liver powder or B_{12} supplement 3 weaned slightly heavier young than did those on the basal ration. These differences did not prove to be statistically significant.

Experiments 10 and 11 were further replications of two of the rations used in experiments 8 and 9. The rats used in experiment 10 were the young from experiment 9 and represented the third successive generation on their respective rations. The rats used in experiment 11 were young from the stock colony which had been placed on the experimental rations at weaning. The results of these two experiments were essentially the same as those of experiments 8 and 9. The higher mortality prior to 48 hours in experiments 10 and 11 was thought to be caused, at least in part, by a high incidence of respiratory infection among the mother rats during these experiments. No differences were found to exist between the supplement-fed lots and the basal, and the higher early mortality was not considered to be due to the ration. In the 4 experiments in which B_{12} supplement 3 was fed, the females receiving this supplement weaned young that averaged 2.1 gm heavier than those from the basal-fed lots. This difference was not large enough for significance when tested by analysis of variance (Snedecor, '46).

A study of the results of experiment 12 shows that the livability up to 48 hours and the number raised to weaning did not differ greatly among the lots. The supplementation of the basal diet with crystalline B_{12} produced only slightly heavier weanling rats. When the APF supplement was added to the basal diet, significant improvement in weaning weights was noted. The addition to the basal ration of both crystalline B_{12} and the APF supplement did not improve the weaning weights over those of young from mothers on the basal ration supplemented with only the APF supplement.

Since the level of protein in the diet has been thought to be related to vitamin B_{12} requirement, basal ration 5 was formulated and used in two experiments. The results of ex-

periments 13 and 14 are found in table 4. The early mortality was slightly higher in the basal-fed lots but the number weaned was essentially the same. The addition of B_{12} supplement 3 to the basal ration significantly improved the weaning weights of the young when these were compared to those of the basal-fed lots.

At no time during the course of this entire investigation has it been possible to demonstrate a high mortality of young during the first 48 hours or prior to weaning. These results are at variance with those of numerous workers (Ross et al., '42; Zucker and Zucker, '48; Hartman et al., '49). Schultze [12] ('50) has recently reported that he could secure satisfactory weight gains from the first generation of rats fed a purified ration containing isolated soybean protein. However, somewhat smaller gains were experienced when the second generation was fed these rations.

It has been our experience that the weaning weight of young from females fed rations presumably low in the "animal protein factor" is consistently less than that observed when the ration is fortified with certain natural and fermentation-by-product concentrates.

Numerous explanations have been advanced in an attempt to rationalize the differences observed by us during the last three years and those found by one of us (O.B.R.) previously and by other workers. There are certain facts that may account for at least some of the differences. First, during these studies generous supplementation with all known dietary nutrients (including choline and folic acid) was practiced. The results of previous studies may have been influenced by a lack of some then-unknown factor. It has recently been shown by Schaefer and others ('50) that the dietary requirements for vitamin B_{12}, choline, and folic acid are interrelated and that one may not be established without considering the concentration of each of the others. Secondly, care was used in all these studies to employ only a high-quality expeller-type

[12] Dr. M. O. Schultze's kindness in sending the authors a copy of his manuscript prior to publication is gratefully acknowledged.

soybean meal from a single source.[13] The variation that is known to occur in natural feeds with respect to other nutrients probably extends to the factor that is necessary for successful reproduction and lactation. It may be that larger amounts of this factor were consistently present in the high-quality meals used in this study. The fact that intestinal synthesis might, under certain conditions, supply adequate amounts of the factor must be recognized. Hartman and his associates ('49) have suggested that the factor is synthesized in adequate amounts when excessively high levels of riboflavin are fed.

SUMMARY

The reproduction and lactation performances of rats fed diets composed principally of corn and soybean meal supplemented with known minerals and vitamins except vitamin B_{12} have been found to be essentially normal. Resorptions, toxemia deaths, or excessive mortality during the first 48 hours after birth, during nursing, or after weaning have not been consistently observed. The weaning weights of the young from females fed supplements of natural and fermentation-by-product concentrates containing vitamin B_{12}, and perhaps other unknown factors, were consistently superior to those of young from females fed unsupplemented rations. Liver powder, concentrates of vitamin B_{12}, a fermentation by-product and an injectible antipernicious anemia extract have all been found to stimulate the growth of nursing rats. The factor involved in this stimulation appears to be soluble in 70% ethanol and less soluble in 95% ethanol.

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[13] See footnote 2, page 535.

liver extract, folic acid and APF supplement used is acknowledged. Thanks are also due to Valley Vitamins, Inc., for donating the alfalfa carotene concentrate used.

LITERATURE CITED

CARY, C. A., AND A. M. HARTMAN 1943–47 Unidentified nutrients. U. S. D. A. Yearbook of Agriculture, Science in Farming, p. 779.

HARTMAN, A. M., L. P. DRYDEN AND C. A. CARY 1949 The role and sources of vitamin B_{12}. J. Am. Diet. Assn., *25:* 929.

ROSS, O. B., P. H. PHILLIPS AND G. BOHSTEDT 1942 The effect of a simplified diet upon reproduction and lactation in swine and in the rat. J. Animal Sci., *1:* 86.

ROSS, O. B., W. E. SWANK, RAY J. OHMAN AND R. W. MACVICAR 1948 The effect of certain supplements upon reproduction and lactation performance of rats fed natural and partially purified rations. Ibid., *7:* 531.

SCHAEFER, A. E., W. D. SALMON, D. R. STRENGTH AND D. H. COPELAND 1950 Interrelationship of folacin, vitamin B_{12}, and choline. Effect of hemorrhagic kidney syndrome in the rat and on growth of the chick. J. Nutrition, *40:* 95.

SCHULTZE, M. O. 1950 Nutritional value of plant materials. I. Growth of rats on purified rations containing soybean protein. Ibid., *41:* 103.

SNEDECOR, G. W. 1946 Statistical Methods Applied to Experiments in Agriculture and Biology. The Iowa State College Press, Ames.

VAN LANDINGHAM, A. H., AND P. B. LYON 1947 Inadequacy of diets containing whole grain cereals supplemented with soybean oil meal for lactation and growth of young rats. Arch. Biochem., *13:* 475.

WATTS, A. B., O. B. ROSS, R. W. MACVICAR AND C. K. WHITEHAIR 1949 The effect of certain supplements upon growth, reproduction and lactation of rats fed partially purified swine rations. J. Animal Sci., *8:* 633.

ZUCKER, L. M., AND T. F. ZUCKER 1948 Zoopherin: A nutritional factor for rats associated with animal protein sources. Arch. Biochem., *16:* 115.

ON THE RELATIVE NUTRITIONAL EFFICIENCY OF SUCROSE AND GLUCOSE IN THE ALBINO RAT [1]

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During the past two or more decades there have been many suggestions that the metabolic paths of glucose and fructose may differ in some respects. A careful study by Wierzuchowski ('26) of the effect of continuous intravenous injection of glucose and fructose for 8 to 16 hours, using normal dogs, indicated the utilization of both sugars, but with clear evidence of essentially different metabolic paths. He cited the earlier work of Bürger ('21), who also found differences in the metabolism of these two sugars. Wierzuchowski's conclusions regarding the utilization of fructose were partly corroborated by Davidson et al. ('36).

Bollman and Mann ('31, '34) reported that fructose was utilized by diabetic dogs during 5 to 10 days of continued feeding of that sugar, although it was thereafter completely converted to glucose and excreted in that form. They expressed the opinion, based on their work with hepatectomized dogs, that the fructose was converted to glucose before being utilized in the peripheral tissues. The opposite conclusion regarding the path of catabolism of fructose was reached by Griffiths and Waters ('36), who reported that fructose is

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utilized as such in the muscle tissues and brain of hepatectomized animals. This conclusion had also been expressed previously by Cori and Cori ('28), based on their extensive studies of the absorption and storage of fructose and glucose and their oxidation in the liver and peripheral tissues.

The independent route of fructose metabolism was again suggested by Wierzuchowski and Sekuracki ('35), who reported that a marked formation of lactic acid occurred in the liver of the dog during the infusion of fructose. The same effect *in vitro* was reported by Dickens and Greville ('32), who used isolated liver tissue and the Warburg technique. Both of these groups stated that the liver did not form lactic acid from glucose. Similar results as to fructose were also obtained by Blatherwick et al. ('40), using rats fed by stomach tube. In their opinion this lactic acid is responsible for the rise in respiratory quotient generally observed after fructose ingestion. The last point is discussed below.

Further instances of the individuality of fructose in metabolism may be cited. Its superior ketolytic (antiketogenic) action over glucose has been shown by Deuel et al. ('32) for human subjects and by Clark and Murlin ('36) for dogs on a high fat diet. A higher absolute rate of glycogen formation from fructose than from glucose has been reported by Cori ('26, '28) when these sugars are given by stomach tube to rats. Based on the actual amounts absorbed (fructose is absorbed only one-half as rapidly as glucose), the difference is more marked, for in two hours 7% of the absorbed glucose and 34% of the absorbed fructose were found as glycogen.

The next and obvious comparison, that of sucrose with glucose, was included in work reported by Feyder and Pierce ('35), who found that after three hours' absorption of concentrated solutions fed to rats by stomach tube, the average rate of glycogenesis in the liver from sucrose was 44% greater than from glucose. They cite the early work of Külz, of Frerichs and of Lusk, who obtained similar results in the

19th century. Feyder and Pierce explain this effect of sucrose by the presence of fructose in the molecule.

The possibility has been suggested, however, that the sucrose molecule behaves differently in metabolism than a mixture of equal parts of glucose and fructose. More than 20 years ago we find Reinhold and Karr ('27) speculating on the fact that sucrose produced a lower rise in their sugar tolerance curves than an equivalent mixture of glucose and fructose. The recent work of Rabinowitch ('45, '47), measuring blood sugar at one-minute intervals, demonstrates the very rapid availability of sucrose when given by mouth to hypoglycemic diabetics. Glucose and fructose, both separately and in equimolar mixtures, are less rapidly effective. He suggests the possibility that sucrose possesses unique physiological properties not shared by an equivalent mixture of glucose and fructose.

A number of workers have discussed the possibility, based on high respiratory quotients, that the administration of fructose may lead to the immediate formation of fat, but there has been little direct evidence in support of this view. A survey of the earlier work on this subject was presented by Bachmann and Haldi ('37), whose own experiments were carefully planned to eliminate previous sources of error, including the extra CO_2 from the lactic acid mentioned above. Their data showed a marked net rise in R.Q., in several cases to above unity, within 15 to 45 minutes after the ingestion of 50 gm of fructose by human subjects in the postabsorptive state. The rise after glucose ingestion was small. Bachmann and Haldi therefore concluded that a portion of the fructose was converted at once into fat. This conclusion was later corroborated by Edwards, Bensley, Dill and Carpenter ('44).

A few feeding experiments on rats, including partial or complete analyses of the carcasses, have also been reported. Feyder ('35) used the paired feeding technique, comparing sucrose and glucose at a level of 68% of the ration. Seven pairs of rats (out of a total of 10 pairs) were analyzed after being fed for 15 to 29 weeks. The gains in weight were

definitely greater in the sucrose animals, and the difference was due principally to a greater deposition of fat. Mitchell, Hamilton and Beadles ('37) compared glucose with sucrose and with fructose, and concluded that differences in the metabolic path of these sugars largely disappear when they are fed as components of a complete diet. They found no significant differences in weight gains or fat deposition, but reported a greater retention of calcium and phosphorus on the sucrose ration than on the glucose one. Support for the idea that fructose favors the deposition of fat was nevertheless indicated in the data from their glucose-fructose comparison. Some fecal losses, probably due to the growth of intestinal bacteria, which might affect the efficiency of utilization of these rations, were also reported by Mitchell et al. In their experiments these losses were significantly greater in the sucrose-fed rats than in those fed glucose.

EXPERIMENTAL

Rations containing 67% of either sucrose or glucose were fed ad libitum to young rats in individual cages in an air-conditioned room (temperature 78° ± 3°F.), using Franke feeding cups and recovering the wasted food on heavy paper under the cages. The rations were identical except for the sugar component (casein [2] 18%; commercial hydrogenated cottonseed oil [3] 5%; dried yeast [4] 5%; salts 4%; and cod liver oil — added daily — 1%).

The sucrose used was commercial granulated sugar, 99.93% pure by polarimetric analysis, containing 0.05% moisture and 0.02% ash. The energy value by bomb calorimeter was 3.931 Cal. per gram. The glucose was commercial cerelose, moisture 9.24%, ash 0.06%, energy value 3.382 Cal. per gram. The latter energy value is the mean of 6 calorimetric determinations, and is equivalent to a moisture-free value of 3.726 Cal. Since the rations were identical except for the

[2] Vitamin-test casein, General Biochemicals, Inc.
[3] Crisco.
[4] "Strain G," Anheuser-Busch, Inc.

sugar, arbitrary values of 3.8 Cal. for the casein and yeast and 9.0 Cal. for the fat were used for calculating the total energy content of the basal ration. On this basis the sucrose ration, as fed, contained 4.048 Cal. per gram, and the glucose ration 3.680 Cal. The difference was due only to the water of crystallization and constitution of the glucose. Water was fed ad libitum.

The rations were fed as nearly as possible according to the daily individual appetite of the animals, without allowing more than a small surplus. Rats which cleaned up the food completely were given 1 or 2 gm more each day until a small amount was left in the cup. The small amount of wasted food on the paper was accurately recovered and saved for the weekly "weigh-back." Weights of food were made to the nearest 0.1 gm in series 2. The rats were apparently in perfect health during the experiments, and the rates of food consumption and gains were uniform.

At the end of the feeding period the animals were starved (except for water) for 24 hours, killed with chloroform, dried at 70°C. and extracted with chloroform to remove the greater part of the fat. The figures for this $CHCl_3$ extract were later incorporated with the data from the analysis of the finely-ground residue of the carcass.[5]

Two experiments are reported in tables 1 and 2. Three preliminary experiments showing the same trends are not reported. The gain in body weight per gram of food is taken as the efficiency of the ration in producing growth. Since this ratio can be only an approximate measure of efficiency, because of the possible intervariations in the fat, protein and water content of the animal body, the actual gains in calories are shown in table 2. These are determined from the analyses of the carcasses (using the values of 4.0 and 9.0 Cal. for protein and fat), minus the estimated calories in the bodies of the weanling rats at the beginning of the experiment. Since females of similar weight and vigor, care-

[5] Grateful acknowledgment is made of the cooperation of Dr. F. E. Hance and Mr. F. R. Van Brocklin.

TABLE 1

Food consumption and weight gains

ANIMAL NO.	LITTER NO.	INITIAL WEIGHT	FINAL WEIGHT	GAIN IN WEIGHT	FOOD CONSUMED	EFFICIENCY OF GAINS [1]
		gm	*gm*	*gm*	*gm*	
			First series (16 weeks)			
			Sucrose ration			
2	..	52	262	210	1431	0.147
3	..	49	242	193	1330	0.145
4	..	48	232	184	1216	0.151
6	..	53	247	194	1294	0.150
7	..	63	248	185	1345	0.138
8	..	68	211	143	1088	0.131
Mean		*55*	*240*	*185*	*1284*	*0.144*
			Glucose ration			
9	..	52	200	148	1259	0.129
10	..	53	206	153	1207	0.139
11	..	48	216	168	1229	0.150
12	..	49	231	182	1333	0.149
13	..	54	222	168	1306	0.141
14	..	52	232	180	1429	0.138
15	..	64	246	182	1514	0.131
16	..	65	216	151	1329	0.125
Mean		*55*	*221*	*166*	*1326*	*0.138*
			Second series (8 weeks)			
			Sucrose ration			
17	1	56	210	154	648.9	0.237
18	2	50	204	154	619.8	0.248
19	2	51	175	124	575.8	0.215
20	2	49	217	168	635.3	0.264
21	3	50	218	168	605.7	0.277
22	3	58	197	139	612.7	0.227
23	4	60	187	127	578.2	0.220
24	5	50	184	134	582.2	0.230
Mean		*53*	*199*	*146*	*607.3*	*0.240*
			Glucose ration			
25	1	56	171	115	642.3	0.198
26	2	51	178	127	641.3	0.219
27	2	50	163	113	609.3	0.204
28	2	49	183	134	623.8	0.237
29	3	48	184	136	682.8	0.220
30	3	60	198	138	724.0	0.211
31	4	58	183	125	625.0	0.220
32	5	51	159	108	619.3	0.192
Mean		*53*	*177*	*124*	*646.0*	*0.213*

[1] The gain in grams per gram of food (growth efficiency) for the glucose ration is shown after correction for water of crystallization and water of constitution of the glucose. This makes the ration isocaloric and comparable on that basis. The food consumed is shown in column 6, however, as it was fed.

fully selected and matched with litter mates in each group, were used in all experiments, any error in the assumption of a similar initial percentage of protein and fat in the bodies of these young rats would have slight effect on the final figures for total caloric gains.

TABLE 2

Composition of carcasses and caloric efficiency [1]

ANIMAL NO.	MOISTURE	FAT AND LIPIDS	CRUDE PROTEIN	ASH	TOTAL CALORIES IN CARCASS	INITIAL CALORIES	GAIN IN CALORIES	FOOD CALORIES INGESTED	CALORIC EFFICIENCY
	gm	gm	gm	gm					
First series (16 weeks)									
Sucrose ration									
2	161.8	46.00	45.25	9.70	595	88	507	5796	0.0875
3	149.6	43.95	41.95	8.60	563	84	479	5386	0.0889
4	136.6	37.30	42.75	8.95	507	83	424	4925	0.0861
6	144.5	42.25	41.55	9.05	546	89	457	5241	0.0872
8	126.9	46.00	37.30	7.65	563	116	447	4406	0.1014
Mean	*143.9*	*43.10*	*41.75*	*8.80*	*555*	*92*	*463*	*5151*	*0.0899*
Glucose ration									
12	140.5	50.50	38.30	7.40	608	84	524	4919	0.1065
13	133.2	30.45	39.85	9.05	433	90	343	4819	0.0712
14	126.2	40.40	40.20	9.15	524	88	436	5273	0.0827
15	157.9	37.15	42.80	8.60	506	109	397	5587	0.0711
16	136.7	32.70	37.50	7.80	444	111	333	4904	0.0679
Mean	*138.9*	*38.25*	*39.75*	*8.40*	*503*	*96*	*407*	*5100*	*0.0798*
Second series (8 weeks)									
Sucrose ration									
17	123.0	27.62	35.03	7.31	389	96	293	2627	0.1115
18	110.6	46.67	31.63	6.22	547	85	462	2510	0.1840
19	97.5	34.13	27.43	5.78	417	87	330	2331	0.1415
20	110.4	57.26	30.34	6.08	637	83	554	2571	0.2155
21	121.9	38.02	35.94	6.92	486	85	401	2453	0.1634
22	112.3	34.03	31.80	6.60	433	99	334	2481	0.1346
23	98.3	43.55	28.63	5.99	506	103	403	2340	0.1723
24	101.6	32.72	29.79	5.84	414	85	329	2356	0.1396
Mean	*109.5*	*39.25*	*31.32*	*6.34*	*479*	*90*	*388*	*2459*	*0.1578*
Glucose ration									
25	94.4	30.75	28.29	5.63	390	96	294	2362	0.1245
26	93.6	38.36	27.55	5.41	455	87	368	2359	0.1560
27	84.0	35.94	26.65	5.07	430	85	345	2242	0.1540
28	96.7	38.19	28.77	5.85	459	83	376	2296	0.1638
29	104.4	30.00	30.34	5.76	391	81	310	2513	0.1234
30	99.4	47.89	31.68	5.96	558	103	455	2663	0.1708
31	101.5	31.02	30.58	6.11	402	99	303	2300	0.1317
32	94.5	18.76	28.90	5.63	284	87	197	2278	0.0865
Mean	*96.1*	*33.86*	*29.10*	*5.68*	*421*	*90*	*331*	*2377*	*0.1390*

[1] Caloric efficiency: net gain in calories over calories ingested.

The differences found in favor of sucrose are slight but quite constant. In the first series (table 1) the growth efficiency is 0.144 ± 0.003 for sucrose and 0.138 ± 0.003 for glucose. The standard error of the mean difference is ± 0.004 for a difference of 0.006. In no case does an individual glucose-fed rat gain in weight as much as the mean gain of the sucrose group. The caloric efficiency (table 2) for the same experiment is 0.0899 ± 0.0028 for sucrose and 0.0798 ± 0.0071 for glucose. The mean difference is 0.0101 ± 0.0076. With the exception of one rat in each group, all efficiency ratios are very concordant, and the highest ratio in the remainder of the glucose group is less than the lowest in the sucrose group.

In the second series, where more animals are involved, the growth efficiency is 0.240 ± 0.008 for sucrose and 0.213 ± 0.005 for glucose. The mean difference is 0.027 ± 0.009, a significant difference. Again each individual gain in the glucose group, as well as each growth efficiency ratio, is less than the corresponding mean of the sucrose group. The caloric efficiency in this experiment (table 2) is 0.1578 ± 0.011 for sucrose and 0.1390 ± 0.010 for glucose. The mean difference is 0.019 ± 0.015. Only two individuals in the glucose group, however, equal or exceed the mean of the sucrose group.

It appears, therefore, that small but measurable differences in nutritional efficiency may exist between sucrose and glucose. Such differences may be largely due to the fructose [6] but may be somewhat involved with the mechanism of dealing with the sucrose molecule as such, as suggested by Reinhold and Karr and by Rabinowitch. Detailed studies on absorption might assist in clearing up the matter but further well-controlled nutritional studies, in other laboratories, are necessary. If extrinsic factors such as intestinal flora or voluntary activity of the animals influence the data, such factors may

[6] The fat content of the carcasses (table 2) indicates, as in Feyder's data, a tendency toward greater fat deposition in the sucrose groups. Metabolism experiments now in progress in this laboratory using radioactive carbon may clear up this matter.

be essential factors in the total environment of the animal. In the case of the experiments reported here, no records were kept of activity but daily observation indicated no noticeable difference between the two groups in this respect.

SUMMARY

There is much evidence in the literature that fructose (and sucrose) follow a somewhat different metabolic path in the animal body than glucose. Two feeding experiments are reported in which sucrose and glucose were fed to rats at a level of 67% of a complete ration. Analysis of the carcasses indicated that the body storage in calories per calorie of food ingested showed a slight difference in favor of the sucrose ration.

LITERATURE CITED

BACHMANN, G., AND J. HALDI 1937 A comparative study of the respiratory quotient following the ingestion of glucose and of fructose as affected by the lactic acid and carbon dioxide changes in the blood. J. Nutrition, *13:* 157.

BLATHERWICK, N. R., P. J. BRADSHAW, M. E. EWING AND S. D. SAWYER 1940 Comparative metabolism of glucose and fructose in the rat. J. Biol. Chem., *136:* 615.

BOLLMAN, J. L., AND F. C. MANN 1931 The physiology of the liver. XIX. The utilization of fructose following complete removal of the liver. Am. J. Physiol., *96:* 683.

———— 1934 Utilization of various carbohydrates by the depancreatized animal. Ibid., *107:* 183.

BÜRGER, M. 1921 The effect on the respiratory exchange of the dog of the intravenous injection of hypertonic solutions of various sugars. Biochem. Zschr., *124:* 1 (original not seen; through Chem. Abstr., *16:* 953).

CLARK, D. E., AND J. R. MURLIN 1936 The effects of glucose, fructose and galactose on ketosis, produced by anterior pituitary extract and by pancreatectomy. J. Nutrition, *12:* 469.

CORI, CARL F. 1926 The fate of sugar in the animal body. III. The rate of glycogen formation in the liver of normal and insulinized rats during the absorption of glucose, fructose and galactose. J. Biol. Chem., *70:* 577.

CORI, C. F., AND G. T. CORI 1928 The fate of sugar in the animal body. VIII. The influence of insulin on the utilization of glucose, fructose, and dihydroxyacetone. Ibid., *76:* 755.

DAVIDSON, J. N., W. O. KERMACK, D. M. MOWAT AND C. P. STEWART 1936 Fructose metabolism in the intact animal. Biochem. J., *30:* 433.

DEUEL, H. J., JR., M. GULICK AND J. S. BUTTS 1932 Studies on ketosis. II. The comparative ketolytic action of glucose, galactose, fructose and sucrose. J. Biol. Chem., *98:* 333.

DICKENS, F., AND G. D. GREVILLE 1932 The metabolism of normal and tumour tissue. VII. The anaerobic conversion of fructose into lactic acid by tumour and adult normal tissues. Biochem. J., *26:* 1546.

EDWARDS, H. T., E. H. BENSLEY, D. B. DILL AND T. M. CARPENTER 1944 Human respiratory quotients in relation to alveolar carbon dioxide and blood lactic acid after ingestion of glucose, fructose, or galactose. J. Nutrition, *27:* 241.

FEYDER, S. 1935 Fat formation from sucrose and glucose. Ibid., *9:* 457.

FEYDER, S., AND H. B. PIERCE 1935 Rates of absorption and glycogenesis from various sugars. Ibid., *9:* 435.

GRIFFITHS, J. P., AND E. T. WATERS 1936 The utilization of fructose in the mammalian organism as shown by experiments on hepatectomized and eviscerated preparations. Am. J. Physiol., *117:* 134.

MITCHELL, H. H., T. S. HAMILTON AND J. R. BEADLES 1937 The comparative nutritive values of glucose, fructose, sucrose and lactose when incorporated in a complete diet. J. Nutrition, *14:* 435.

RABINOWITCH, I. M. 1945 Short period blood sugar time curves following ingestion of sucrose. Ibid., *29:* 99.

———— 1947 Metabolism of sucrose. II. Am. J. Digest. Dis., *14:* 315.

REINHOLD, J. G., AND W. G. KARR 1927 Carbohydrate utilization. II. Rate of disappearance of various carbohydrates from the blood. J. Biol. Chem., *72:* 345.

WIERZUCHOWSKI, M. 1926 Intermediary carbohydrate metabolism. I. Influence of insulin on levulose and glucose intravenously administered. Ibid., *68:* 631.

WIERZUCHOWSKI, M., AND F. SEKURACKI 1935 Spaltungs-, Oxydations- und Energieumsatz beim Hunde. I. Bildung und Beseitigung der Milchsäure in den Organen beim Hungern, sowie während der Oxydation von Galaktose, Glucose und Maltose. Biochem. Zschr., *276:* 91. II. Organ-quellen und -mündungen der Milchsäure während der Umsetzung der Fruktose, auf Grund der Kontrollversuche mit Inosit, Acetaldehyd und Natriumlactat. Ibid., *276:* 112.

VITAMIN B_{12} PRODUCTION BY MICROORGANISMS ISOLATED FROM POULTRY HOUSE LITTER AND DROPPINGS

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Only a limited number of reports have been published to date on the production of vitamin B_{12} by microorganisms. The present authors have made an extensive study of the production of vitamin B_{12} by microorganisms isolated from poultry house litter and droppings in connection with an investigation of the requirements of growing chicks for this factor under different management practices, and some of the observations are presented here.

A very extensive report on the production of vitamin B_{12} by microorganisms has been made by Lewis et al. ('49) of the U. S. Department of Agriculture Western Regional Research Laboratory. These investigators found *Bacillus megatherium* to be the most active producer of a large number of the vitamin B_{12}-producing microorganisms that were investigated. This organism was reported to produce 0.8 parts per million of vitamin B_{12} in a 12-hour batch fermentation in a medium containing sucrose, ammonium nitrogen, yeast extract and inorganic salts. Yields of 150 to 200 mμg of vitamin B_{12} per milliliter of whole culture were produced after 4 to 6 days of incubation in shake flasks at 25°C.

Both zinc and cobalt were found by Lewis et al. ('49) to be important ingredients of the vitamin B_{12} production me-

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dium. Although omission of zinc or cobalt was without effect on growth, it reduced the vitamin B_{12} produced to two-thirds in the case of zinc and one-tenth to one-fourth in the case of cobalt.

Ansbacher et al. ('49b) have used a medium composed mainly of soybean oil meal for synthesizing the "animal protein factor" (principally vitamin B_{12}) by microorganisms. They report that yeast, although an excellent source of the vitamin B complex, normally has no "animal protein factor" (APF) activity, but that it may be supplied with high APF potency by certain fermentation processes. Using the growth stimulation produced by 5% fish meal in a basal all-plant diet as the unit of comparison in chick assays, products containing 20 times as much APF activity as fish meal were obtained by fermentation processes without resorting to chemical purification procedures.

Ansbacher and Hill ('49a) reported that the chemical reactions involving vitamin B_{12} production seemed to involve choline, since choline was either destroyed or utilized during the fermentation process that produced relatively high amounts of the APF complex.

Stokstad et al. ('49) have reported the production of the "animal protein factor" plus another growth-promoting factor with *Streptomyces aureofaciens.*

Wolf, Wood and Folkers ('49) have isolated crystalline vitamin B_{12} from a strain of *Streptomyces griseus* which produces the antibiotic streptomycin.

Short and Briggs ('48) have reported that culture filtrates of *Lactobacillus casei, Streptococcus faecalis R,* and *Escherichia coli* have low vitamin B_{12} potencies.

EXPERIMENTAL

The microorganism isolations used for this study were obtained from pour plates. These pour plates were made by using a measured quantity of aqueous extracts from poultry house litter and poultry droppings and mixing this extract with the media in which microorganisms were grown for iden-

tification and counting. Streak plates were made from broth cultures prepared from isolated colonies on the pour plates. When isolations were obtained in a pure state by the procedure indicated, as evidenced by colony appearance, staining, and microscopic examination, a tube of vitamin B_{12} production medium was inoculated for vitamin B_{12} production study.

TABLE 1

Modified California[1] *vitamin* B_{12} *production media*

INGREDIENTS	AMOUNT PER LITER
Sucrose	50 gm
Yeast extract	2.5 gm
Citric acid	0.5 gm
Ammonium sulfate $(NH_4)_2SO_4$	4.0 gm
Dibasic anhydrous sodium phosphate (Na_2HPO_4)	2.0 gm
Potassium chloride (KCl)	0.8 gm
Magnesium nitrate $(Mg(NO_3)_2 + 6H_2O)$	500 mg (50 p.p.m. Mg)
Dibasic calcium phosphate $(CaHPO_4 \cdot 2H_2O)$	215 mg (50 p.p.m. Ca)
Manganous sulfate $(MnSO_4 \cdot H_2O)$	45 mg (15 p.p.m. Mn)
Ferrous sulfate $(FeSO_4 \cdot 7H_2O)$	25 mg (5 p.p.m. Fe)
Zinc dust	5 mg (5 p.p.m. Zn)
Cobalt chloride $(CoCl_2 + 6H_2O)$	8 mg (2 p.p.m. Co)

The zinc dust is dissolved in concentrated HCl and the rest of the ingredients in distilled water.

Adjusted to pH 7.0 with 20% KOH and distilled water added to make up to 1,000 ml.

[1] U.S.D.A. Western Regional Research Lab. Mimeo. Report AIC-254.

The medium used for vitamin B_{12} production studies is shown in table 1. It differs only slightly from the medium used by Lewis et al. ('49) in that it contains the same amounts of cations of the various minerals and the same amounts of sucrose and yeast extract but not the same anions in all cases.

The size of the sample medium inoculated was 10 ml, which were placed in an 18 × 150 mm culture tube with a non-absorbent cotton plug and sterilized for 15 min. at 15 lb. pressure prior to inoculation.

Duplicate sets of inoculated tubes were incubated at 30°C. and at 37°C. for 48 to 72 hours with two or three shakings by hand during this period. One milliliter of the broth was then assayed for its vitamin B_{12} activity by using *Lactobacillus leichmannii* (American Type Culture Collection 4797) and following the procedure described by Skeggs et al. ('48) modified by the inclusion of 0.1% of sodium thioglycollate in the assay medium and the use of a pH of 5.7 instead of 6.8 for most assays. Growth was measured turbidimetrically by means of a Coleman Universal spectrophotometer set at a wavelength of 610 mμ. Crystalline vitamin B_{12} [2] was used as the standard.

Culture samples were usually autoclaved prior to removal of the assay sample. This was especially true where curds, pellicles or mycelia were present to interfere with securing a representative sample of the culture. Autoclaving has been shown by Lewis et al. ('49) to release the vitamin B_{12} from the cells into the supernatant, and it was also thought that autoclaving was desirable in order to secure a more representative aliquot and to stop growth.

One to 10 and one to 100 broth dilutions were used for assaying the more potent culture samples. Other dilutions were used when necessary to permit the finding of the most sensitive assay range.

Several known organisms, such as *Bacillus megatherium, Streptomyces griseus, Streptomyces aureofaciens, Bacillus subtilis, Staph. aureus,* and *Eschericha coli* were tested along with the unknown isolations.

RESULTS AND DISCUSSION

One hundred and forty-two separate isolations were studied for their vitamin B_{12} production, including bacteria, yeast and molds. This group without question included some duplications, since no special effort was made to identify the organisms unless they were found to produce a large amount

[2] Cobione.

of vitamin B_{12} in comparison with *Bacillus megatherium, Streptomyces griseus,* or *Streptomyces aureofaciens.*

A summary of the vitamin B_{12} production studies is shown in table 2. It was found that only 2.8% of the 142 isolated organisms studied produced no detectable quantity of vitamin B_{12} (less than 0.01 mμg of vitamin B_{12} per milliliter of broth), 32.3% produced between 0.01 and 0.10 mμg per milliliter,

TABLE 2

Vitamin B_{12} production in vitro by microorganisms isolated from poultry house litter and droppings. (Also a comparison with some known microorganisms)

ORGANISMS	NON-DETECTABLE QUANTITY	0.01 to 0.10 [1]	0.10 to 0.40 [1]	Over 0.40 [1]	HIGHEST PRODUCTION TEMPERATURE	
					37°C.	30°C.
A. *Known*						
Bacillus subtilis		x				x
Staph. aureus		x			x	
Escherichia coli, 1		x			x	
Escherichia coli, 2		x			x	
B. megatherium, O.S.U. strain				x		x
B. megatherium, WRRL no. B938				x	x	
Streptomyces griseus				x		x
Streptomyces aureofaciens				x		x
B. *Unknown* (Total of 142)						
Classification on basis of production	4	46	32	60	52	86
Per cent of total	2.8	32.3	22.5	42.2	37.7	62.3

[1] Vitamin B_{12} production in millimicrograms per milliliter of broth.

22.5% produced between 0.10 and 0.4, and 42.2% more than 0.4 under the conditions employed.

Effects of incubation temperature on vitamin B_{12} production

Vitamin B_{12} production studies at different incubation temperatures showed that approximately two-thirds of the isolated organisms produced vitamin B_{12} best at 30°C. (62.3%)

and one-third (37.7%) at 37°C. This is in agreement with the observation (Halbrook et al., '50a) that poultry droppings stored at 30°C. for one week increased in vitamin B_{12} activity by more than 900% as compared to only 390% when they were stored at 37°C.

Vitamin B_{12} production with and without choline in the medium

The vitamin B_{12} production medium was modified in one instance to include 0.2% choline chloride in order to note the effect of this modification on vitamin B_{12} production, since Ansbacher et al. ('49b) had reported either use or destruction of choline by microorganisms in vitamin B_{12} production.

The results of this study showed that the addition of choline to the production media at the rate of 0.2% reduced the vitamin B_{12} production of 14 of the organisms as much as 33% when compared to that of similar media without added choline. Only two organisms showed a slight increase in production of the vitamin. Since no effort was made to vary the level of choline chloride in the medium, it is possible that some other level would have given more favorable results.

Final pH of vitamin B_{12} production broth

In order to understand better what was occurring in vitamin B_{12} production, the final pH of the vitamin B_{12} production broth after incubation for 72 hours at 30° and 37°C. was determined. The original medium with a pH adjusted to 7.0 was changed in most cases by the organism tested toward the acid side. Only 9 of the 33 organisms tested in this phase of the study produced an alkaline reaction at one or both temperatures. The highest pH was 7.7 and the lowest 3.1. The average was 6.0 after incubation at 30°C. and 6.3 after incubation at 37°C.

Kinds of organisms producing vitamin B_{12} activity

An effort was made to classify some of the higher vitamin B_{12}-producing organisms. Among these were found both

Gram negative and Gram positive bacteria, both rod and cocci forms, and also yeasts and molds. It can be said, therefore, that all of these classes of microorganisms contribute to the sum total of vitamin B_{12} production in poultry house litter and droppings. However, yeast and molds probably play a minor role, as is shown by the almost complete inhibition of these microorganisms upon incubating droppings for one week at 30° and 37°C. (Halbrook et al., '50b), while vitamin B_{12} production increased.

One of the best producing organisms, isolated and identified in our studies as no. 48, produced more vitamin B_{12}, as determined by microbiological assay, than *Bacillus megatherium* (WRRL B938), *Streptomyces griseus* (O.S.U. strain), or *Streptomyces aureofaciens* (NRRL 2209) under our test conditions.

This organism is a non-motile small Gram negative rod, isolated from an eosin methylene blue plate. The rods occur singly. The organism produces on agar large, white, smooth, opaque colonies with a rancid fecal odor. The colonies were opalescent by transmitted light and creamy white on agar slope. In nutrient broth the growth was turbid with sediment. In thioglycollate medium the growth was very turbid with a heavy ring. No liquefaction of gelatin occurred either in stab or on Frazier's gelatin plates. It reduced nitrates to nitrites. Tryptophan broth showed no indole. Acid was produced in litmus milk in 48 hours and a curd was formed at the end of a week. The organisms produced both acid and gas in sucrose, lactose, dextrose, xylose, arabinose and mannitol. Koser's citrate was positive and also acetylmethyl-carbinol. Lead acetate agar showed gas with a positive-negative test for hydrogen sulfide. The results of these tests coincide in general with the description given in Bergey's manual ('48) for *Aerobacter aerogenes*, and our organism has been tentatively identified as a strain of this species of bacteria.

SUMMARY

1. Only 2.8% of 142 isolations of microorganisms from poultry house litter and droppings which were studied for

vitamin B_{12} production produced no detectable quantity (less than 0.01 mμg per milliliter of broth) of vitamin B_{12} activity as determined by microbiological assay with *Lactobacillus leichmannii* (A.T.C.C. 4797); 32.3% produced between 0.01 and 0.10; 22.5% between 0.1 and 0.4; and 42.2% more than 0.4 mμg per milliliter of broth.

2. Vitamin B_{12} production studies at 30° to 37°C. showed that 62.3% of the isolated organisms produced better at 30°C. than at 37°C., and 37.7% produced better at 37°C.

3. The addition of 0.2% of choline chloride to the vitamin B_{12} production medium reduced the total production of vitamin B_{12} of 14 of the organisms studied by 33%. Only two of the 14 produced better (but only slightly) with the addition of choline chloride.

4. Thirty-three organisms, which were selected as being the better vitamin B_{12} producers, produced an average change in the vitamin B_{12} production medium from an original pH of 7.0 to 6.0 after incubation for 72 hours at 30°C. and to 6.3 after incubation for the same period at 37°C. Only 9 of the organisms produced an alkaline reaction at one or both temperatures. The lowest pH recorded was 3.1 and the highest was 7.7.

5. Among the better vitamin B_{12}-producing organisms isolated from poultry house litter and droppings were yeast, molds, and many classes of bacteria. One organism was isolated which produced more vitamin B_{12} as measured microbiologically than either *Bacillus megatherium, Streptomyces aureofaciens* or *Streptomyces griseus* under our test conditions. This organism has been tentatively identified as a strain of *Aerobacter aerogenes.*

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LITERATURE CITED

ANSBACHER, S., AND HARLEY H. HILL 1949a Selective synthesis of the APF complex by fermentation. Abstract of paper presented at the Amer. Chem. Soc. meeting, Atlantic City, N. J., Sept. 18–23.

ANSBACHER, S., HARLEY H. HILL, J. M. TIEMAN, H. F. DOWNING AND JOHN H. CALDWELL, JR. 1949b Microbially synthesized APF (animal protein factor). Fed. Proc., *8:* 180.

BERGEY'S MANUAL OF DETERMINATIVE BACTERIOLOGY 1948 R. S. Breed, E. G. D. Murray and A. P. Hitchens, ed. Williams and Wilkins, Baltimore, 6th ed.

HALBROOK, E. R., T. S. SUTTON AND A. R. WINTER 1950a The effect of management on the vitamin B_{12} content of poultry house litter. II. As determined by microbiological assay. Poultry Sci., in press.

HALBROOK, E. R., A. R. WINTER AND T. S. SUTTON 1950b The microflora of poultry house litter and droppings. In preparation.

LEWIS, J. C., K. IJICHI, N. S. SNELL AND J. A. GARIBALDI 1949 Fermentation process for production of vitamin B_{12}. Bureau of Agr. and Indust. Chem., U. S. D. A., Mimeo. Pub. 254, p. 7.

SHORT, MARY S., AND G. M. BRIGGS 1948 The effect of dissociation in *Lactobacillus lactis* cultures on the requirement for vitamin B_{12}. J. Biol. Chem., *176:* 1463.

SKEGGS, H. R., L. D. WRIGHT, J. W. HUFF AND D. K. BOSSHARDT 1948 The use of *Lactobacillus leichmannii* in the microbiological assay of the "animal protein factor." Ibid., *176:* 1459.

STOKSTAD, E. L. R., T. H. JUKES, J. PIERCE, A. C. PAGE, JR. AND A. L. FRANKLIN 1949 The multiple nature of the "animal protein factor." Ibid., *180:* 647.

WOLF, D. E., T. R. WOOD AND K. FOLKERS 1949 Studies on vitamin B_{12}. Abstract of paper presented before the Amer. Chem. Soc. meeting, Atlantic City, N. J., Sept. 18–23.

UTILIZATION OF VITAMIN A BY TURKEY POULTS

I. CRYSTALLINE CAROTENE, CRYSTALLINE VITAMIN A ACETATE AND "BLACK COD" LIVER OIL[1]

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FOUR FIGURES

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INTRODUCTION

In view of the length of time that vitamin A and its precursors have been under investigation, and considering their recognized importance in poultry nutrition, there is surprisingly little agreement regarding the vitamin A requirement of either chickens or turkeys. Reports in the literature indicate that the requirement of the turkey for vitamin A is from 2 to 4 times as high as that of the chicken, based on intake per unit weight of diet. The chicken in turn is reported to require from 8 to 10 times as much vitamin A as the rat per unit of body weight. There must be some basic reason for these reported wide differences in the utilization of vitamin A. Are they the result of differences in actual requirements or are they reflections of the degree of utilization?

Guilbert and Hinshaw ('34) reported that chickens were able to utilize carotene from freshly-cut and dehydrated al-

[1] Authorized for publication on March 16, 1950, as paper no. 1589 of the journal series of the Pennsylvania Agricultural Experiment Station. The data in this paper were presented at the 37th Annual Meeting of the Poultry Science Association at Fort Collins, Colorado, June 1948.

falfa 4 to 5 times more efficiently than turkeys, as measured by liver storage of vitamin A. Record, Bethke and Wilder ('37) studied different sources of vitamin A and observed that, for growth, chicks utilized carotene as a source of vitamin A as efficiently as the rat. They found no difference in efficiency between carotene and fish liver oil, unit for unit. Johnson, Carrick and Hauge ('48) reported that chicks which received alfalfa meal stored more vitamin A in their livers than chicks fed the calculated equivalent levels of the vitamin as supplied by fish oil. Nestler, Derby and DeWitt ('48) reported that bobwhite quail utilized vitamin A acetate, natural vitamin A ester, vitamin A alcohol and crystalline carotene in decreasing order of efficiency as measured by storage of vitamin A in the liver at 10 weeks of age. Carotene in alfalfa meal was used about as efficiently as crystalline carotene.

The purpose of the investigation here reported was to try to discover why attempts to measure the vitamin A requirement of turkey poults have given such diverse results, and possibly to gain some insight as to why turkeys are reported to require so much more vitamin A than chickens. The studies described in this report include two experiments in which the utilization of carotene and vitamin A were compared in the turkey poult. Crystalline carotene was compared with vitamin A from "Black Cod" liver oil in experiment 1 and with crystalline vitamin A acetate in experiment 2. The bases of comparison were the rate of growth and the amount of vitamin A found in the liver. Additional data were collected on yellow pigments in the liver and on both vitamin A and yellow pigments in the blood plasma.

EXPERIMENTAL

The day-old White Holland turkey poults used in both experiments were obtained from the same breeding flock maintained by the Department of Poultry Husbandry. This

strain of turkeys is characterized by medium size and, when reared in confinement for replacement stock, has an average body weight of about 950 gm at 8 weeks of age.

The poults were kept through the 4th week of age in electrically heated starter batteries with hardware cloth floors. At the beginning of the 5th week they were transferred to grower batteries without heating units, located in a room where the temperature was thermostatically controlled. Water and feed were provided ad libitum.

For the first two weeks of each experiment all poults were fed the vitamin A-deficient diet which had the following percentage composition: 30.1 ground wheat, 14 soybean oil meal (expeller-processed), 10 wheat bran, 10 wheat standard middlings, 10 ground oats, 6 dried whey, 6 commercial casein, 8 brewers' dried yeast, 3.5 pulverized limestone, 1.9 cottonseed oil, 0.3 iodized salt, 0.1 manganese sulfate and 0.1 D-activated animal sterol. The chemical composition of the diet determined by analysis was: protein ($N \times 6.25$) 22.6%, ether extract 4.4%, crude fiber 4.5%, ash 7.1%, calcium 1.8%, phosphorus 0.8%, and moisture 10%. The two-week period on the vitamin A-deficient diet was used to deplete the poults of vitamin A. The experiments were conducted for 8 weeks; a depletion period of 2 weeks, and 6 weeks on the supplemented diets.[2]

The vitamin A potencies of "Black Cod" liver oil and crystalline vitamin A acetate in cottonseed oil were determined colorimetrically by the Carr-Price ('26) method referred to below. The potency of crystalline carotene in cottonseed oil was calculated from the weight of carotene used, 0.6 μg being considered equivalent to 1 I.U. This value was multiplied by 0.95, since the crystalline carotene con-

[2] The supplements were obtained from the following sources: crystalline carotene (90% beta-, 10% alpha-) in 1-gm ampules from General Biochemicals, Inc.; "Black Cod" liver oil, Lot B-16487 with a potency of about 533,000 I.U. per gram, from the R. P. Scherer Corp.; crystalline vitamin A acetate in 1-gm ampules from Distillation Products, Inc. Cottonseed oil, from the Buckeye Cotton Oil Co., was used as a means of incorporating the supplements into the basal diet.

tained 10% alpha-carotene, which is considered to be half as active as beta-carotene biologically. Care was exercised to minimize losses in the vitamin A and carotene preparations and in the diets after supplements were added.

In experiment 1, 310 poults were used in 13 groups. Poults in groups 1 to 6 inclusive received crystalline carotene incorporated into the basal diet in quantities increasing from 950 to 30,400 I.U. of vitamin A per pound of diet.[3] Poults in groups 7 to 12 inclusive received "Black Cod" liver oil as the source of vitamin A, in quantities increasing from 1,000 to 32,000 I.U. of vitamin A. Poults in group 13 received no supplement and served as negative controls.

In experiment 2, 375 poults were used in 17 groups. Poults in groups 1 to 8 received crystalline carotene at levels of from 238 to 30,400 I.U. of vitamin A. Poults in groups 9 to 16 received vitamin A acetate at levels of from 250 to 32,000 I.U. Poults in group 17 served as negative controls.

In both experiments poults were weighed individually at 7-day intervals, but to save space only average group weights at 5 and 8 weeks of age are given in the tables, because at those ages the poults had received the supplemented diets for 3 and 6 weeks, respectively. In experiment 1, at the end of the 8th week, the poults were killed and their livers used for vitamin A determinations. In experiment 2, poults were taken from groups 6, 7, 8, 14, 15 and 16 for vitamin A and yellow pigment determinations in the livers, at 7-day intervals, through the 8th week of age. Vitamin A and yellow pigments were determined in blood plasma at the 5th, 6th, 7th and 8th weeks of age in experiment 2. All vitamin A and yellow pigment determinations were made on composite samples representing from 2 to 8 poults per group. Only two determinations were made on samples composed of as few as two poults. The composite liver samples were prepared in a Waring Blendor.

[3] The concentration of vitamin A henceforth will be expressed in International Units of vitamin A per pound of diet. In order to avoid repetition, the phrase "per pound of diet" will be omitted.

Determination of vitamin A in liver was carried out by the Carr-Price ('26) colorimetric method, as described in Methods of Vitamin Assay of the Association of Vitamin Chemists ('47). An Evelyn photoelectric colorimeter, previously standarized against the U.S.P. reference standard, was used to measure vitamin A (Dann and Evelyn, '38). Inasmuch as the U.S.P. vitamin A reference standard has been accepted as the international vitamin A reference standard all vitamin A data have been expressed in International Units. Vitamin A and the yellow pigments in blood plasma were determined according to the method described by Kimble ('39). The total yellow pigments and the yellow pigments which passed through the chromatographic column were evaluated by using crystalline beta-carotene (90% beta-, 10% alpha-) as the standard and measuring the light transmission with a 440-mμ filter by the aid of an Evelyn photoelectric colorimeter. The adsorbent used for the chromatographic column, about 15 mm in diameter and 150 mm in length, was a mixture of one part by weight of MgO [4] with three parts of Hyflo Super-Cel. While it is highly improbable that the pigmentation measured was due to beta-carotene, it had been found previously that this particular column was effective in separating beta-carotene from the other pigments when a 3% acetone in petroleum ether extract of green plant tissue was used. The beta-carotene passed through in the eluate, while the other pigments were retained on the column.

RESULTS AND DISCUSSION

In experiment 1, poults that received 3,800 I.U. of vitamin A in the form of crystalline carotene attained approximately normal body weight. Supplementation with carotene above this level stimulated little additional growth. Table 1 records the average body weights of the poults.

Poults that received "Black Cod" liver oil, however, attained normal body weight at a level of 2,000 I.U. The higher

[4] Micron brand, no. 2641.

intakes did not result in any increase in the rate of growth. In fact, upon examination of the data in table 1 it appears that higher intakes may have caused a slight depression in the growth rate.

TABLE 1

Average body weights of turkey poults at 5 and 8 weeks of age

VITAMIN A PER LB. OF DIET	EXPERIMENT 1				EXPERIMENT 2			
	Group no.	No. of poults at 8th wk.	5th wk.	8th wk.	Group no.	No. of poults at 8th wk.	5th wk.	8th wk.
I.U.			*gm*	*gm*			*gm*	*gm*
	Crystalline carotene				*Crystalline carotene*			
238					1	0 [2]		
475					2	2	371	674
950	1	16	397	884	3	2	352	768
1,900	2	16	425	1,010	4	7	405	927
3,800	3	16	436	1,095	5	11	402	1,004
7,600	4	16	455	1,105	6	10	409	977
15,200	5	16	458	1,141	7	8	406	1,046
30,400	6	16	453	1,111	8	5	404	1,090
	"Black Cod" liver oil				*Crystalline vitamin A acetate*			
250					9	3	391	665
500					10	6	373	884
1,000	7	16	445	1,041	11	11	412	1,016
2,000	8	16	460	1,155	12	15	427	1,065
4,000	9	16	458	1,109	13	11	420	1,043
8,000	10	16	462	1,078	14	9	386	879
16,000	11	16	417	1,055	15	9	388	935
32,000	12	16	437	1,098	16	7	426	1,024
None	13	0 [1]			17	0 [2]		

[1] All 22 poults in group 13 were dead by the 5th week.

[2] All poults in groups 1 and 17 (14 in each) were dead by the 5th week.

Figure 1 illustrates a comparison of the growth response among three levels of crystalline carotene and two levels of "Black Cod" liver oil. Between the 7th and 8th weeks, poults that received crystalline carotene levels of 950 and 1,900, and a "Black Cod" liver oil level of 1,000 I.U., showed a decrease in the rate of growth; whereas, those that received the crystalline carotene level of 3,800, and a "Black Cod"

liver oil level of 2,000 I.U., did not show any such decrease. This would indicate that the crystalline carotene levels below 3,800 and the "Black Cod" liver oil level below 2,000 I.U. were not adequate to promote normal growth in turkey poults to the 8th week of age. In comparing the two vitamin A supplements on the basis of body weight increase it is obvious

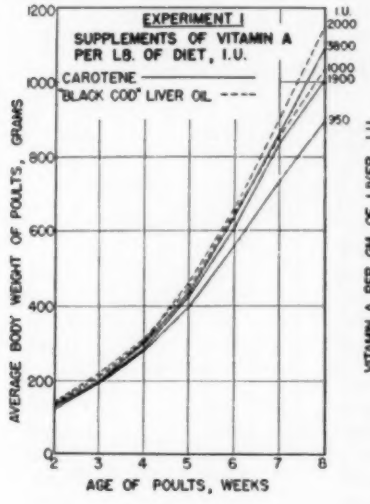

Figure 1

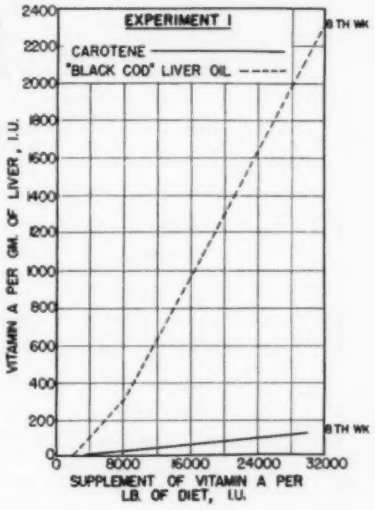

Figure 2

Fig. 1 Body weights of poults that received different levels of vitamin A from two sources.

Fig. 2 Liver storage of vitamin A in poults that received different levels of vitamin A from two sources.

from figure 1 that "Black Cod" liver oil at 2,000 I.U. supported a faster rate of growth than crystalline carotene at 3,800 I.U. The same relationship holds true between the "Black Cod" liver oil level of 1,000 and the crystalline carotene level of 1,900. Thus it appears that on the basis of growth rate and in terms of International Units of vitamin A activity this lot of "Black Cod" liver oil was about twice as efficient in promoting growth as crystalline carotene.

Poults in the negative control group (no. 13) showed retardation of growth at the third week and no growth between the 3rd and 4th weeks of age, when most of the deaths occurred. Out of 22 poults, only 5 remained alive at the beginning of the 5th week. All poults developed a staggering gait but only a few showed symptoms of xerophthalmia or had

TABLE 2

Experiment 1

Vitamin A in livers of poults at 8 weeks of age [1]

GROUP NO.	SUPPLEMENT		UNITS PER GM OF LIVER	UNITS PER LIVER
	Source	Vitamin A per lb. of diet		
		I.U.	*I.U.*	*I.U.*
1	Crystalline carotene	950	[2]	[2]
2		1,900	[2]	[2]
3		3,800	6	128
4		7,600	19	414
5		15,200	54	1,272
6		30,400	129	3,040
7	"Black Cod" liver oil	1,000	[2]	[2]
8		2,000	10	228
9		4,000	102	2,322
10		8,000	316	6,822
11		16,000	975	21,167
12		32,000	2,329	53,799

[1] Amounts of vitamin A in livers at hatching and at second week were 92 and 2.7 I.U. per gram, respectively.

[2] No measurable amount was found.

urate deposits in the kidneys or ureters. No histological examination of the tissues was carried out.

The vitamin A liver storage in experiment 1 showed great differences between corresponding levels of the two supplements. Poults that received 950 and 1,900 I.U. as crystalline carotene and 1,000 I.U. in the form of "Black Cod" liver oil stored no measurable amount of vitamin A in the liver. Table 2 records the average amounts of vitamin A in International Units per gram and per liver determined at the 8th

week of age. At intakes higher than those required to support approximately normal growth, poults fed "Black Cod" liver oil stored 16 to 18 times more vitamin A than poults that received corresponding levels of crystalline carotene. Figure 2 illustrates this difference in liver storage.

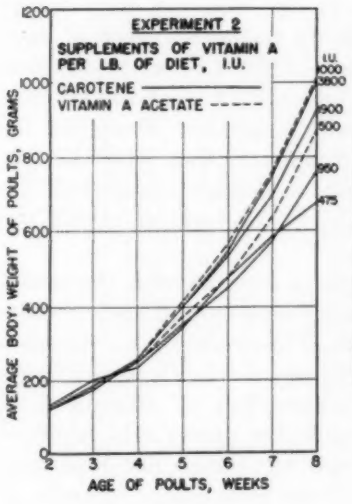

Figure 3

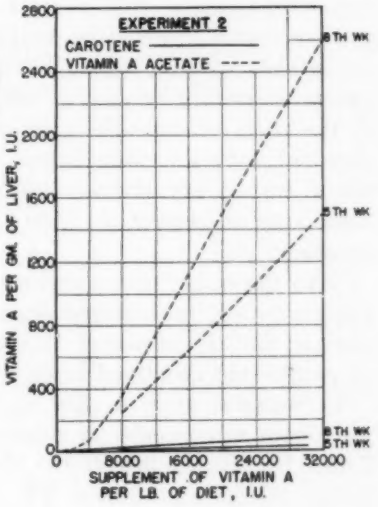

Figure 4

Fig. 3 Body weights of poults that received different levels of vitamin A from two sources.

Fig. 4 Liver storage of vitamin A in poults that received different levels of vitamin A from two sources.

In experiment 2, crystalline vitamin A acetate and crystalline carotene were compared with respect to efficiency in promoting growth and liver storage of vitamin A in poults. Table 1 records the average body weights at 5 and 8 weeks of age. Under the conditions of this experiment, 2,000 I.U. from crystalline vitamin A acetate were sufficient to support normal growth. Higher levels of this supplement appeared to cause a slight depression in the growth rate.

Poults that received crystalline carotene required more than 2,000 I.U. for normal growth. Even the highest intakes of crystalline carotene did not suppress growth, but rather resulted in a slight increase in the growth rate. Poults in the negative control group were all dead by the 5th week.

The data presented in figure 3 permit a comparison of the two supplements with respect to the efficiency with which growth was supported in poults, up to the 8th week of age, by intakes of 475, 950, 1,900 and 3,800 I.U. as crystalline carotene, and by intakes of 500 and 1,000 I.U. of the vitamin in the form of crystalline vitamin A acetate. From the individual curves in this figure, it appears that 1,000 I.U. in the form of crystalline vitamin A acetate supported the same rate of growth as 3,800 I.U. in the form of crystalline carotene.

Near the end of the second week, in experiment 2, the poults contracted an unidentified disease which caused some deaths during the experimental period. This reduced the number of poults that remained in each group at the 8th week of age.

In experiment 2, weekly determinations of vitamin A in the livers were made on representative poults that received 7,600, 15,200 and 30,400 I.U. from crystalline carotene, and 8,000, 16,000 and 32,000 I.U. from crystalline vitamin A acetate. Table 3 records the average vitamin A storage per gram of liver to 8 weeks of age. These data show that at the end of the 8th week the poults which received crystalline vitamin A acetate stored from 20 to 30 times more vitamin A in the liver than the poults which received corresponding levels of crystalline carotene. With both supplements there appears to be, with increasing age and with increasing levels of intake, a definite increase in the amount of vitamin A stored in the liver. Only 7 exceptions occurred in 38 observations.

Table 3 also records the average amounts of vitamin A stored per liver. These values were obtained by multiplying the average amount of vitamin A per gram of liver by the average liver weight. When liver stores are expressed as

TABLE 3

Experiment 2

Vitamin A in livers of poults from hatching to 8 weeks of age [1]

VITAMIN A PER LB. OF DIET	GROUP NO.	VITAMIN A PER LIVER (AND PER GRAM OF LIVER)[2]					
		3rd wk.	4th wk.	5th wk.	6th wk.	7th wk.	8th wk.
I.U.		*I.U.*	*I.U.*	*I.U.*	*I.U.*	*I.U.*	*I.U.*
				Crystalline carotene			
238	1						[3]
475	2						[4]
950	3						[4]
1,900	4						[4]
3,800	5						99 (4)
7,600	6	[4]	[4]	162 (12)	93 (6)	273 (13)	423 (14)
15,200	7	52 (9)	83 (9)	165 (13)	342 (23)	884 (35)	1,217 (42)
30,400	8	100 (18)	244 (29)	314 (24)	1,912 (131)	2,991 (144)	2,377 (86)
				Crystaline vitamin A acetate			
250	9						[4]
500	10						[4]
1,000	11						47 (2)
2,000	12						985 (28)
4,000	13						2,189 (76)
8,000	14	694 (120)	2,322 (270)	3,372 (248)	5,915 (397)	8,643 (437)	10,179 (369)
16,000	15	1,580 (316)	4,861 (605)	8,109 (654)	14,152 (976)	23,215 (1,155)	34,877 (1,144)
32,000	16	3,617 (723)	9,436 (1,329)	17,628 (1,507)	31,278 (2,406)	57,367 (2,706)	78,731 (2,607)

[1] Amounts of vitamin A in livers at hatching and at first and second weeks were 152 (127), 106 (44), and 8 (3) I.U. per liver, respectively.

[2] The values in parentheses represent vitamin A per gram of liver.

[3] All poults in group 1 were dead by the 5th week.

[4] No measurable amount was found.

the total amount of vitamin A per liver, the 7 irregularities which occur in table 3 expressed as International Units of vitamin A per gram of liver are reduced to two.

Figure 4 illustrates the average liver storage of vitamin A in experiment 2, at the 5th and 8th weeks of age; it shows also an increase in storage, from the 5th to 8th week, with the use of both supplements. The difference in liver storage of the vitamin resulting from feeding the two supplements is shown clearly in this figure.

Crystalline vitamin A acetate produced 22 to 33 times as much vitamin A storage at the 8th week as did crystalline carotene, on the basis of total vitamin A per liver. This suggests a possible limitation in the ability of the poult to absorb or to convert the ingested crystalline carotene to vitamin A.

Beginning with the 5th week, in experiment 2, vitamin A in blood plasma was also determined at weekly intervals in groups 6, 7, 8, 14, 15 and 16 and in all groups at the 8th week of age (table 4). In poults which received crystalline carotene a rapid increase in blood plasma vitamin A took place up to a level of 7,600 I.U.; whereas in poults which received crystalline vitamin A acetate, this rapid increase ceased at an intake of about 2,000 I.U. of the supplement. Although there was an increase of the vitamin A content in the blood plasma of poults that received more than 7,600 I.U. from crystalline carotene and more than 2,000 I.U. from crystalline vitamin A acetate, this increase was small in comparison with the increase in the levels of the supplements.

While there were fluctuations in the amounts of vitamin A found in the blood plasma at different ages, poults that received crystalline vitamin A acetate had about twice as much vitamin A in their blood plasma as those which received corresponding levels of crystalline carotene.

It is possible that the stage of digestion may have a direct influence on the blood plasma content of vitamin A. In an effort to reduce variations that might result from this and

TABLE 4

Experiment 2

Vitamin A and total yellow pigments in the blood plasma of poults to 8 weeks of age [1]

VITAMIN A PER LB. OF DIET	GROUP NO.	AVERAGES PER 100 ML OF BLOOD PLASMA			
		5th wk.	6th wk.	7th wk.	8th wk.
I.U.		*I.U.*[2] *μg* [3]	*I.U.*[2] *μg* [3]	*I.U.*[2] *μg* [3]	*I.U.*[2] *μg* [3]
				Crystalline carotene	
238	1				[4]
475	2				39 (24)
950	3				58 (24)
1,900	4				94 (26)
3,800	5				186 (26)
7,600	6	223 (39)	227 (30)	239 (27)	263 (35)
15,200	7	243 (37)	262 (23)	243 (24)	293 (32)
30,400	8	287 (38)	352 (39)	357 (32)	334 (29)
				Crystalline vitamin A acetate	
250	9				40 (21)
500	10				86 (24)
1,000	11				144 (26)
2,000	12				310 (26)
4,000	13				374 (26)
8,000	14	463 (14)	374 (12)	410 (15)	429 (23)
16,000	15	596 (13)	627 (8)	577 (10)	627 (11)
32,000	16	759 (10)	679 (7)	691 (7)	623 (12)

[1] Total yellow pigments in the blood plasma at hatching and at second week were 701 and 32 μg per 100 ml, respectively.
[2] International Units of vitamin A.
[3] The values in parentheses represent color equivalents in micrograms of beta-carotene.
[4] All poults in group 1 were dead by the 5th week.

related influences, all poults had access to their respective supplemented diets until they were killed.

In experiment 2, the total yellow pigments in the livers of the poults and those yellow pigments which passed through the chromatographic column were determined. Table 5 records, in micrograms per gram, the total yellow pigments found in the livers, from hatching to 8 weeks of age. These pigments were determined by using beta-carotene (90% beta-, 10% alpha-) as the standard and measuring the light transmission with a 440-mμ filter by the aid of an Evelyn photoelectric colorimeter. Although it is highly probable that the yellow pigments were carotenoids other than beta-carotene, their color equivalents are expressed in terms of beta-carotene.

The livers of poults at hatching were found to contain relatively large quantities of yellow pigments, with an average value of 17.3 μg per gram. The pigments decreased rapidly, however, to 2.9 μg per gram at two weeks of age.

From the 3rd to the 8th week of age, yellow pigments were found in the livers of all poults, regardless of the type of vitamin A supplement received. Even though the quantities of these pigments were small, they may be related to the diet and may play a rôle in body function. Values obtained at the 8th week showed a slight increase in the amount of yellow pigments in the liver as the intake of the supplement increased in poults which received crystalline carotene; whereas the reverse was true in poults which received crystalline vitamin A acetate.

Table 5 also records the values of the yellow pigments which passed through the chromatographic column, from the livers of the poults in experiment 2. There was an increase in these pigments from the livers of poults that received increased quantities of crystalline carotene. To a lesser degree the same was true of poults that received increased quantities of crystalline vitamin A acetate.

Table 4 also records the yellow pigment values in the blood plasma of the poults in experiment 2. These values, ex-

TABLE 5

Experiment 2

Total yellow pigments, and yellow pigments which passed through the chromatographic column, in livers of poults from hatching to 8 weeks of age [1]

VITAMIN A PER LB. OF DIET	GROUP NO.	YELLOW PIGMENTS PER GRAM OF LIVER					
		3rd wk.	4th wk.	5th wk.	6th wk.	7th wk.	8th wk.
I.U.		*μg*	*μg*	*μg*	*μg*	*μg*	*μg*
				Crystalline carotene			
238	1						[3]
475	2						0.36 ( )[4]
950	3						0.54 (0.12)[2]
1,900	4						0.46 (0.15)
3,800	5						0.55 (0.25)
7,600	6	1.45 (0.31)[2]	0.93 (0.15)[2]	0.68 (0.13)[2]	0.83 (0.26)[2]	0.47 (0.21)[2]	0.60 (0.32)
15,200	7	1.50 (0.39)	0.75 (0.18)	0.79 (0.16)	0.70 (0.37)	0.46 (0.29)	0.63 (0.39)
30,400	8	1.20 (0.12)	0.84 (0.30)	0.94 (0.46)	0.88 (0.52)	0.83 (0.58)	0.88 (0.58)
				Crystalline vitamin A acetate			
250	9						0.53 (0.06)
500	10						0.41 (0.10)
1,000	11						0.46 (0.15)
2,000	12						0.37 (0.14)
4,000	13						0.45 (0.16)
8,000	14	0.93 ( )[4]	0.39 (0.23)	0.41 (0.15)	0.39 (0.12)	0.40 (0.09)	0.32 (0.18)
16,000	15	1.15 (0.26)	0.35 (0.30)	0.33 (0.06)	0.37 (0.14)	0.43 (0.09)	0.31 (0.18)
32,000	16	1.25 ( )[4]	0.51 (0.23)	0.30 (0.15)	0.45 (0.16)	0.42 (0.14)	0.32 (0.22)

[1] Yellow pigments in the livers at hatching and at first and second weeks were 17.3 (1.6), 10.5 (1), and 2.9 (0.2) μg per gram, respectively. All values represent color equivalents in micrograms of beta-carotene.

[2] The values in parentheses represent yellow pigments which passed through the chromatographic column.

[3] All poults in group 1 were dead by the 5th week.

[4] No measurable amount was found.

pressed in micrograms per 100 ml of blood plasma, were high at hatching but decreased markedly by the second week of age. From the 5th to the 8th week of age the yellow pigments in the blood plasma of poults which received crystalline carotene appeared to be present in about the same quantity in all groups. In poults that received crystalline vitamin A acetate, however, there was a decrease of yellow pigments in the blood plasma of poults in groups 15 and 16, which received 16,000 and 32,000 I.U. of the vitamin. This is probably a manifestation of the phenomenon reported by Deuel et al. ('43) and by Rubin and Bird ('46). Deuel et al. reported that ingestion by hens of large quantities of vitamin A from shark liver oil resulted in marked suppression of the carotenoid pigment content of the egg yolk, blood, body fat and liver. Rubin and Bird reported that pigmentation in the shanks of chicks was inhibited by a high intake of crystalline vitamin A.

It is suspected that the yellow color found in the liver and in blood plasma is not due to the presence of beta-carotene but perhaps to a mixture of other carotenoids. Data on the light absorption characteristics of these pigments will be reported in a succeeding paper.

SUMMARY

The comparative utilization of carotene and of two forms of vitamin A by turkey poults up to 8 weeks of age was studied in two series of feeding tests. Gain in body weight and the amount of vitamin A present in the liver were employed as indices of utilization. Vitamin A in the blood plasma and yellow pigments in both liver and blood plasma were also determined. The results of these studies permit the following conclusions:

1. On the basis of International Units of vitamin A activity, "Black Cod" liver oil, lot B-16487, was found to be approximately twice, and crystalline vitamin A acetate approximately 4 times, as efficient in supporting normal growth in poults as the corresponding levels of crystalline carotene.

2. At intakes higher than those required to support normal growth much more vitamin A was stored in the liver when either crystalline vitamin A acetate or "Black Cod" liver oil was fed than when the diet contained corresponding levels of crystalline carotene.

3. Poults which received crystalline vitamin A acetate had about twice as much vitamin A in the blood plasma as those which received corresponding levels of crystalline carotene.

4. Yellow pigments in the livers and in the blood plasma of poults were found to be high at hatching but decreased rapidly during the first two weeks of life.

5. High intakes of crystalline vitamin A acetate were found to depress the total yellow pigments in the liver and to a greater extent those in the blood plasma.

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LITERATURE CITED

ASSOCIATION OF VITAMIN CHEMISTS, INC. 1947 Methods of Vitamin Assay. Interscience Publishers, Inc., New York, pp. 19–68.

CARR, F. H., AND E. A. PRICE 1926 Colour reactions attributed to vitamin A. Biochem. J., *20:* 497–501.

DANN, W. J., AND K. A. EVELYN 1938 The determination of vitamin A with the photoelectric colorimeter. Biochem. J., *32:* 1008–1017.

DEUEL, H. J., JR., M. C. HRUBETZ, F. H. MATTSON, M. G. MOREHOUSE AND ALAN RICHARDSON 1943 Studies on carotenoid metabolism. IV. The effect of vitamin A intake on the carotenoid and vitamin A content of the eggs, liver, blood and body fat of hens. J. Nutrition, *26:* 673–685.

GUILBERT, H. R., AND W. R. HINSHAW 1934 Vitamin A storage in the livers of turkeys and chickens. Ibid., *8:* 45–56.

JOHNSON, E. L., C. W. CARRICK AND S. M. HAUGE 1948 The vitamin A requirements of young chickens. Poultry Sci., *27:* 308–314.

KIMBLE, M. S. 1939 The photocolorimetric determination of vitamin A and carotene in human plasma. J. Lab. Clin. Med., *24:* 1055–1065.

NESTLER, R. B., J. V. DERBY AND J. B. DEWITT 1948 Storage by bobwhite quail of vitamin A fed in various forms. J. Nutrition, *36:* 323–329.

RECORD, P. R., R. M. BETHKE AND O. H. M. WILDER 1937 The vitamin A requirements of chicks with observations on the comparative efficiency of carotene and vitamin A. Poultry Sci., *16:* 25–33.

RUBIN, MAX, AND H. R. BIRD 1946 The apparent antagonism between vitamin A and carotenoids in the fowl. Science, *103:* 584–586.

A COMPARISON OF TECHNIQUES USED IN DIGESTIBILITY STUDIES WITH DAIRY CATTLE[1]

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Various methods have been proposed to simplify the procedure and to reduce the amount of labor and expense involved in the determination of digestibility coefficients. One of the most popular of these methods is to feed an indigestible material and then calculate digestibility coefficients from the ratios of the nutrients and the indigestible material in the feed and feces. With this method the total collection of feces is unnecessary, as the digestibility can be computed from selected portions of the feces if the nutrients and indigestible material are evenly distributed.

Bergeim, one of the first to use an inert chemical substance in the determination of digestibility coefficients, used iron oxide successfully in 1926. Heller, Breedlove and Likely ('28) obtained fairly accurate results with iron oxide when using rats as experimental animals. Moore and Winter ('34) reported that iron oxide did not give accurate results when used in bovine digestibility investigations. Knott, Murer and Hodgson ('36) found iron oxide unsatisfactory for measuring digestibility coefficients with cattle. Hale, Duncan and Huffman ('39) stated that variations in the amount of iron oxide

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passing through the digestive tract make it unreliable as a measure of digestibility.

Diverse results have also been observed when lignin is used as an indicator in the determination of digestibility coefficients. Hale, Duncan and Huffman ('39) claimed lignin ratios were not reliable as a measure of total digestion. Forbes and Swift ('43) found lignin to vary in digestibility from negative values to plus 29%. Crampton and Jackson ('44) stated that lignin cannot be relied on to indicate the trends in digestibility. Ellis et al. ('46) reported that lignin was not digested in any significant amount. Swift et al. ('47) claimed the lignin ratio method of determining digestibility was highly valid. Forbes and Garrigus ('48) applied the lignin ratio technique to the study of the digestion of pasture forages with apparent success.

The value of silica as a reference material in digestibility studies is also controversial. Some claim that the dust in the air (barns) or the contamination of the feed with dirt (silica) interferes seriously with the determination of digestibility coefficients when silica is used as an indicator. Gallup and Kuhlman ('36) reported that since approximately 15% of the silica was metabolized, it was not a trustworthy indicator. Druce and Willcox ('49) stated that the amount of silica recovered in the feces was too variable to be of service in digestion studies. Skulmowski et al. ('43) reported adequate agreement between digestibility coefficients determined by silica ratios and those obtained by the standard total collection procedure.

Chromium oxide has also been used as an indicator of digestibility in studies with cattle. Edin et al. ('44), Andersen ('34), and Skulmowski et al. ('43) have all used it with great success. On the other hand, Kreula ('47) found it difficult to recover all the chromium oxide fed — regaining only 97.5%.

Reid and associates ('50) have used chromogenic substances, which are indigestible, as reference material for the indirect measurement of digestibility. The feed and feces

are extracted with 85% acetone, and the resulting colored solution read in a spectrophotometer at 406 mμ.

The objective of the experiment reported in this paper was to determine the reliability of digestibility coefficients obtained by the lignin or chromium oxide method as compared with those secured by the conventional method.

EXPERIMENTAL

A direct comparison of the digestibility coefficients obtained by using either lignin or chromium oxide as an indicator was made with those calculated by the standard total collection method. All comparative determinations by the different methods were made from composite samples representing the total collection of feces. In other words, this work is not a comparison of the indicator method, as such, in which only a portion of the feces is collected and analyzed, with other procedures, but an examination of the three techniques with a view to determining the differences due solely to method. It was felt that this was a necessary step in evaluating the indicator procedures in order to distinguish between errors due to method and those due to sampling.

The forage fed in this experiment was second-crop alfalfa, all grown on the same field at Beltsville, cut in the quarter-bloom stage, and conserved in three different ways: (1) as silage; (2) as field-cured hay; and (3) as barn-cured hay. The crop was cut on July 9 and 10, 1947. The alfalfa silage and barn-cured hay were stored either on the same day they were cut or on the day after cutting. The field-cured hay was rained on several times and badly damaged. It was in the swath and windrow for an average of 131 hours, whereas the alfalfa silage and barn-cured hays averaged 10 and 38 hours, respectively, in the swath and windrow.

Three producing cows were used in this experiment. All three were crossbreds, designated as X-10, X-54, and X65. X-54 gained around 100 lb. in weight during the three months on trial; the weights of the other two cows remained stationary.

The daily ration of each cow consisted of 8 lb. of grain, 20 lb. of corn silage and 15 lb. of alfalfa hay or 40 lb. of alfalfa silage. The total digestible nutrient (TDN) value of the ration was adjusted to the weight and milk production of the animals by altering slightly the amounts of corn silage and alfalfa fed. The grain mixture consisted of yellow corn meal 44%, wheat bran 22%, ground oats 22%, soybean oil meal 11%, and iodized salt 1%.

Eighteen digestion trials of approximately 9 days' duration were carried out by each of the three methods: total collection, lignin, and chromium oxide. Six digestion trials by each of the three methods were conducted with each of the three types of forage. All experimental periods in which a change of ration occurred were preceded by a preliminary feeding period of 10 days.

If both chromium oxide and lignin pass through the bovine digestive tract unabsorbed, after a sufficient length of time (10 days) the daily intake of the indicator substance should equal the daily output in the feces. This being true, one is then able to calculate the digestion coefficient of any component of the ration by means of the following formula:

$$\text{Digestibility} = 100 - 100\left(\frac{\%\text{ Inert chemical in feed}}{\%\text{ Inert chemical in feces}} \times \frac{\%\text{ Nutrient in feces}}{\%\text{ Nutrient in feed}}\right).$$

PROCEDURES

Proximate analyses of feeds and feces were conducted essentially according to the procedures described by the Association of Official Agricultural Chemists ('45). Moisture determinations were made in a vacuum oven at a negative pressure of approximately one atmosphere overnight.

Lignin determinations were performed by the method of Ellis, Matrone and Maynard ('46).

The chromium oxide method used is reported here in detail, since Edin's method was modified to some extent in order to attain greater accuracy and better recoveries.

Baker's C.P. chromium oxide was mixed with flour (Cr_2O_3 15%). The resulting mixture was made into a hard bread by

wetting, mixing to a stiff dough, and baking. The hard bread was dried and then ground with a medium mesh screen in a Wiley mill. The chromium oxide-flour mixture was added to the grain so that the cows received approximately 15 gm Cr_2O_3 per day, or 100 gm of the chromium flour.

The proper amount of Cr_2O_3 in the sample for analyses was found to be 20 to 40 mg. The proper amount of sample was weighed into nickel crucibles and ashed until free of carbon; 8 hours at 600°C. was sufficient. The crucibles had a volume of 125 ml, a height of 6 cm, and a diameter of 6 cm. When free of carbon, the ashed material was fused with a flux consisting of 4 gm of potassium hydroxide, 2.7 gm of sodium hydroxide, and 5.8 gm of a mixture of 138 gm (1 mol.) of potassium carbonate, 106 gm (1 mol.) of water-free sodium carbonate, 202 gm (2 mol.) of potassium nitrate, and 170 gm (2 mol.) of sodium nitrate. The total 12.5 gm of the fusion mixture was weighed out and added to each crucible, care being taken to limit the water uptake of the fusion mixture.

The crucibles were then heated on a hot plate until the mixture became liquid and swirled at 5-minute intervals during an additional heating period of 20 minutes.

While the material was still hot a nickel wire, 1 mm thick and 15 cm long and formed into a spiral at one end, was placed in the fused material. After the mass had solidified, the crucible was quickly heated with a gas flame to melt the material around the walls so it could be lifted out with the nickel wire.

The melted material was placed in a 600-ml glass beaker, the crucible was thoroughly washed with warm distilled water, and the washings were transferred into the beaker. The material was then dissolved in 150 to 200 ml of water. To the solution was added 1 ml of 30% H_2O_2 for reduction of any trace of 6 valent Mn to the 4 valent state. When the solution had boiled for one minute, it was filtered through a sintered glass filter funnel into a 500-ml flat-bottomed flask in order to remove the precipitated manganese dioxide. Then 8.3 ml of concentrated H_2SO_4 (ACS) and 2.5 ml of H_2O_2 (30%)

TABLE 1

Digestibility coefficients on alfalfa silage

	PERIOD I					PERIOD II				
	Dry matter	Crude protein	Crude fiber	N-free extr.	Ether extr.	Dry matter	Crude protein	Crude fiber	N-free extr.	Ether extr.
	Cow 65					*Cow 65*				
Total Collection	60.2	61.8	43.0	71.3	64.5	61.7	64.9	44.1	72.6	64.4
Chromium Oxide	60.4	62.1	43.3	71.5	64.7	61.8	65.0	44.2	72.7	64.5
Lignin	61.5	63.0	44.9	72.3	65.7	62.0	65.1	44.5	72.8	64.6
	Cow 54					*Cow 54*				
Total Collection	60.9	62.9	41.8	72.9	67.0	61.0	64.9	43.0	72.0	65.6
Chromium Oxide	60.8	62.9	41.8	72.9	67.0	61.8	65.6	44.1	72.6	66.3
Lignin	61.3	63.3	42.5	73.2	67.4	62.3	66.0	44.9	73.0	66.8
	Cow 10					*Cow 10*				
Total Collection	61.2	62.8	43.5	72.1	69.6	62.2	65.8	43.6	73.1	65.5
Chromium Oxide	60.6	62.2	42.6	71.6	69.2	62.6	66.2	44.1	73.4	65.8
Lignin	61.7	62.3	42.8	71.7	69.3	62.3	65.8	43.6	73.1	65.5
	Average of 3 cows, Period I					*Average of 3 cows, Period II*				
Total Collection	60.8	62.5	42.8	72.1	67.0	61.6	65.2	43.6	72.6	65.2
Chromium Oxide	60.6	62.4	42.6	72.0	67.0	62.1	65.6	44.1	72.9	65.5
Lignin	61.5	62.9	43.4	72.4	67.5	62.2	65.6	44.3	73.0	65.6

	Average of 6 trials, 3 cows, 2 periods				
	Dry matter	Crude protein	Crude fiber	N-free extr.	Ether extr.
Total Collection	61.2	63.8	43.2	72.4	66.1
Chromium Oxide	61.4	64.0	43.4	72.4	66.2
Lignin	61.8	64.2	43.8	72.7	66.6
	Average of 18 trials, 3 cows, 2 periods, 3 methods				
	61.5	64.0	43.5	72.5	66.3

were added, and the solution heated to and kept at boiling temperature for 5 minutes in order to destroy nitrites present. When the solution had cooled, 2 ml H_2O_2 (30%) and 34.5 ml 6.75 N NaOH were added and the mixture heated again and boiled for 5 minutes to oxidize the reduced chromium salts to the chromate. Without interrupting the boiling, 5 ml of 5% nickel nitrate were added, and the boiling was continued for 5 minutes longer to destroy excess H_2O_2 completely. After cooling, the solution was acidified slowly with 18.3 ml concentrated H_2SO_4, which had been boiled previously for 4 hours. When the solution had cooled, 10 ml of a 10% potassium iodide (ACS) solution were added and the solution titrated with 0.05 N $Na_2S_2O_3$. A 1% starch solution was used as an indicator. The statistical treatment of the data has been in accord with the general principles expressed by Snedecor ('46).

RESULTS

Table 1 gives the digestibility coefficients of the various components in the ration containing alfalfa silage. The close agreement among the three methods of determination is clearly indicated by the data for each component, both for period I and also for its replicate period II.

Tables 2 and 3 show the same data when the field-cured alfalfa and barn-cured alfalfa were fed. Again the consistent agreement among the three methods is indicated by the data for all the components of the ration.

Tables 4 and 5 show the percentages of ingested lignin and chromium oxide recovered in the feces. The recoveries obtained in this experiment were considered satisfactory. The percentage of recovery of the chromium oxide showed less variation than that of the lignin.

An analysis of variance conducted on each component of the ration revealed the following facts. There were no significant differences between cows. There were highly significant differences due to methods used in harvesting and preserving the

TABLE 2

Digestibility coefficients on field-cured alfalfa

	PERIOD III					PERIOD IV				
	Dry matter	Crude protein	Crude fiber	N-free extr.	Ether extr.	Dry matter	Crude protein	Crude fiber	N-free extr.	Ether extr.
	Cow 65					*Cow 65*				
Total Collection	60.3	62.5	46.4	71.0	56.1	57.5	60.1	42.1	69.7	54.2
Chromium Oxide	60.3	62.5	46.7	71.9	55.9	57.4	60.0	41.9	69.6	54.1
Lignin	59.7	61.9	45.8	70.4	55.1	58.6	61.1	43.5	71.4	55.3
	Cow 54					*Cow 54*				
Total Collection	59.8	62.6	43.5	71.8	56.6	59.7	62.5	46.2	70.2	55.4
Chromium Oxide	60.0	62.7	43.3	71.9	56.8	58.5	61.4	44.7	69.3	54.2
Lignin	59.5	62.3	43.0	71.6	56.3	58.4	61.3	44.5	69.2	54.0
	Cow 10					*Cow 10*				
Total Collection	60.7	63.1	46.1	71.9	59.0	58.8	59.9	45.1	69.4	54.2
Chromium Oxide	59.4	61.8	44.4	70.8	57.5	59.0	60.1	45.3	69.5	54.4
Lignin	58.3	60.8	42.9	70.1	56.3	57.5	58.7	43.4	68.4	52.8
	Average of 3 cows, Period III					*Average of 3 cows, Period IV*				
Total Collection	60.3	62.7	45.3	71.6	57.2	58.7	60.8	44.5	69.8	54.6
Chromium Oxide	59.9	62.3	44.8	71.5	56.7	58.3	60.5	44.0	69.5	54.2
Lignin	59.2	61.7	43.9	70.7	55.9	58.2	60.4	43.8	69.7	54.0

	Average of 6 trials, 3 cows, 2 periods				
	Dry matter	Crude protein	Crude fiber	N-free extr.	Ether extr.
Total Collection	59.5	61.8	44.9	70.7	55.9
Chromium Oxide	59.1	61.4	44.4	70.5	55.4
Lignin	58.7	61.0	43.8	70.2	55.0
	Average of 18 trials, 3 cows, 2 periods, 3 methods				
	59.1	61.4	44.4	70.5	55.4

TABLE 3

Digestibility coefficients on barn-cured alfalfa

	PERIOD V					PERIOD VI				
	Dry matter	Crude protein	Crude fiber	N-free extr.	Ether extr.	Dry matter	Crude protein	Crude fiber	N-free extr.	Ether extr.
	Cow 65					*Cow 65*				
Total Collection	63.5	69.9	46.3	73.6	51.2	64.2	68.5	48.5	74.4	53.4
Chromium Oxide	62.6	69.2	45.1	73.0	50.1	64.9	69.1	49.4	74.9	54.3
Lignin	63.5	69.9	46.3	73.6	51.2	62.8	67.2	46.4	73.4	51.5
	Cow 54					*Cow 54*				
Total Collection	63.7	69.5	47.2	74.2	52.0	63.3	67.2	47.2	73.4	57.0
Chromium Oxide	64.3	70.0	48.0	74.6	52.7	64.0	67.8	48.2	73.9	57.8
Lignin	62.4	68.3	45.2	73.2	50.2	61.9	66.0	45.2	72.4	55.4
	Cow 10					*Cow 10*				
Total Collection	63.6	70.0	43.7	74.4	52.0	63.0	66.7	47.3	73.9	50.7
Chromium Oxide	63.5	69.9	43.5	74.3	51.8	63.0	66.7	47.3	73.9	50.7
Lignin	61.9	68.6	40.9	73.1	49.6	61.9	65.7	45.7	73.1	49.2
	Average of 3 cows, Period V					*Average of 3 cows, Period VI*				
Total Collection	63.6	69.8	45.7	74.1	51.7	63.5	67.5	47.7	73.9	53.7
Chromium Oxide	63.5	69.7	45.5	74.0	51.5	64.0	67.9	48.3	74.2	54.3
Lignin	62.6	68.9	44.1	73.3	50.3	62.2	66.3	45.8	73.0	52.0

	Average of 6 trials, 3 cows, 2 periods				
	Dry matter	Crude protein	Crude fiber	N-free extr.	Ether extr.
Total Collection	63.6	68.6	46.7	74.0	52.7
Chromium Oxide	63.8	68.8	46.9	74.1	52.9
Lignin	62.4	67.6	45.0	73.2	51.2
	Average of 18 trials, 3 cows, 2 periods, 3 methods				
	63.3	68.3	46.2	73.8	52.3

alfalfa. There were no significant differences due to method of determining digestibility; that is, among the total collection, lignin, and chromium oxide procedures.

TABLE 4

Recovery of lignin in per cent

PERIOD	COW 65	COW 54	COW 10	AVERAGE OF 3 COWS
I	103.4	101.2	98.7	101.1
II	100.8	103.4	100.3	101.5
III	98.5	99.4	94.3	97.4
IV	102.3	97.1	97.2	98.9
V	99.6	96.4	95.4	97.1
VI	97.4	96.4	97.1	97.0
	100.3	99.0	97.2	98.8

TABLE 5

Recovery of chromium oxide in per cent

PERIOD	COW 65	COW 54	COW 10	AVERAGE OF 3 COWS
I	100.6	99.9	98.4	99.6
II	100.3	102.1	101.0	101.1
III	99.9	100.5	96.7	99.0
IV	99.7	97.3	100.5	99.2
V	97.8	100.9	99.4	99.4
VI	101.8	101.9	100.0	101.2
	100.0	100.4	99 3	99.9

DISCUSSION

The results of this investigation showed that there were no significant differences between the digestion coefficients obtained by the total collection method and those secured by the ratio procedure using chromium oxide and lignin. In this investigation the ratios with chromium oxide and lignin were calculated from analyses of the composite samples obtained from total collection of the feces.

The results further show that it was possible to recover practically 100% of the chromium oxide and lignin fed, which

indicates that there is no loss during the process of digestion. Therefore, chromium oxide can be considered an indigestible substance suitable for the ratio technique since it can be fully recovered by the chemical procedures used in this study. Lignin, as used in this experiment, falls in the same category.

Until further data are collected, however, the use of lignin should be adopted with some reserve, since it is not a chemical entity of a definite composition. The make-up of lignin in plants may vary, depending on the species and maturity of the plant, which might in turn affect the chemical determination of lignin. Similarly, these factors might also affect the digestibility of lignin.

As a matter of fact, Pazur and De Long ('48) reported that lignin in the earlier stages of growth was more readily metabolized by ruminants than the lignin in more mature plants. If the above finding is true, this variability in the digestion of lignin would interfere with the determination of digestibility by lignin ratios, providing the experimental animals were fed herbage of varying ages. The variation in the digestibility of lignin due to differences in the stage of maturity of forages is now under investigation.

According to calculations based on a ration of grain, corn silage, and alfalfa, the digestibility of the barn-cured alfalfa ration was significantly higher than that of either the alfalfa-silage ration or the field-cured alfalfa ration in dry matter, in protein, in crude fiber, and in nitrogen-free extract.

On the same basis — that is, the ration as a whole — the alfalfa silage was very much higher in digestibility than the field-cured alfalfa in dry matter, protein, nitrogen-free extract, and ether extract. There was no significant difference between these two rations in digestibility of crude fiber.

Alfalfa silage was also significantly higher in the digestibility of ether extract than the barn-cured alfalfa ration. So was the field-cured alfalfa.

The fact that no significant differences were found between the total collection method and the lignin or chromium oxide methods demonstrates that the latter methods have definite

possibilities of reducing the labor and expense involved in the determination of digestibility coefficients.

Although errors due solely to method have been proved to be not significant, it still remains to be established how important the errors of sampling may be. It is also necessary to devise a system of sampling feces that will enable investigators, in conducting digestion trials, to attain the same degree of accuracy in determining digestibility coefficients by taking a few samples of feces as can be obtained by collecting all the feces.

Preliminary investigation (unpublished data) has shown that one feces sample per period is not enough for practical use in digestibility calculations. Nor is the average digestibility of a single sample per period from three cows adequate.

By using ratio techniques it should be possible to conduct digestion trials with animals in ordinary cow stalls without a 24-hour watch or special apparatus for the collection of feces. It is, of course, necessary to have an accurate sample of the feed consumed so that the ratios can be calculated. Similarly, it is necessary to have an accurate record of feeds consumed, and of any uneaten portions, should there be such.

The ratio technique should prove a valid method for use with other types of animals than cattle, including small animals. It should also be applicable to all nutrients, including vitamins, wherever apparent digestibility figures are required.

SUMMARY

1. A statistical analysis showed no significant difference between digestibility coefficients obtained by the use of lignin and chromium oxide ratios and those calculated by the standard total collection procedure. This held true for all nutrients in the ration.

2. The use of indigestible materials such as lignin and chromium oxide shows promise of saving much of the time, labor, and expense involved in the present cumbersome method of conducting digestion trials.

3. Although errors due to method have been proved to be not significant, the problem of avoiding significant errors due to sampling methods still remains to be solved.

4. The data indicate complete recovery of lignin and chromium oxide. Therefore, these indigestible materials are suitable for digestive studies.

5. The barn-cured alfalfa ration was significantly higher than the other two alfalfa rations in digestibility of dry matter, protein, crude fiber, and nitrogen-free extract. In ether-extract digestibility, the barn-cured alfalfa ration was inferior to the other two.

6. The alfalfa silage ration was significantly higher than the field-cured alfalfa ration (rain damaged) in digestibility of dry matter, protein, nitrogen-free extract, and ether extract.

LITERATURE CITED

ANDERSEN, A. C. 1934 Die Futtermittelanalyse und die Bestimmung der Verdaulichkeit der Futtermittel. Skand. Arch. Physiol., *69:* 33–58.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1945 Official and Tentative Methods of Analysis, Washington, D. C., 6th ed., Chap. XXVII, pp. 404–420.

BERGEIM, O. 1926 Intestinal chemistry. IV. A method for the study of food utilization or digestibility. J. Biol. Chem., *70:* 29–33.

CRAMPTON, E. W., AND I. R. C. JACKSON 1944 Seasonal variation in chemical composition of pasture herbage and the relation to its digestibility by steers and sheep (Pasture studies. XXVI). J. Animal Sci., *3:* 333–339.

DRUCE, E., AND J. S. WILLCOX 1949 The application of modified procedures in digestibility studies. Empire J. Exp. Agr., *17:* 188–192.

EDIN, H., G. KIHLEN AND S. NORDFELDT 1944 A summarized description of "Edin's indicator method" for the determination of the digestibility of feeds and feed mixtures. Lantbrükshögskolans Hüsdjürsförsöksanstalt, *12:* 166–171.

ELLIS, G. H., G. MATRONE AND L. A. MAYNARD 1946 A 72 percent H_2SO_4 method for the determination of lignin and its use in animal nutrition studies. J. Animal Sci., *5:* 285–297.

FORBES, E. B., AND R. W. SWIFT 1943 Conditions Affecting the Digestibility and the Metabolizable Energy of Feeds for Cattle. Penna. State College Bulletin 452, pp. 1–34.

FORBES, R. M., AND W. P. GARRIGUS 1948 Application of a lignin ratio technique to the determination of the nutritive intake grazing animals. J. Animal Sci., *7:* 373–382.

GALLUP, W. D., AND A. H. KUHLMAN 1936 The composition and digestibility of mung-bean silage, with observations on the silica-ratio procedure for studying digestibility. J. Agr. Res., *52:* 889–894.

HALE, E. B., C. W. DUNCAN AND C. F. HUFFMAN 1939 Rumen digestion studies. Proc. Soc. Amer. Soc. Animal Prod., *32:* 389–393.

HELLER, V. G., C. H. BREEDLOVE AND W. LIKELY 1928 A comparison of the Bergeim and standard methods of determining coefficients of utilization with suggested modifications. J. Biol. Chem., *LXXIX:* 275–282.

KANE, E. A., W. C. JACOBSON AND L. A. MOORE 1949 Digestibility studies on dairy cattle: A comparison of the total collection methods: conventional versus chromium oxide and lignin techniques. J. Animal Sci., *8:* 623.

KNOTT, J. C., H. K. MURER AND R. E. HODGSON 1936 The determination of the apparent digestibility of green and cured grass by modified procedures. J. Agr. Res., *53:* 553–556.

KREULA, M. S. 1947 Absorption of carotene from carrots in man and the use of the quantitative chromic oxide indicator method in the absorption experiments. Biochem. J., *41:* 269–273.

MOORE, L. A., AND O. B. WINTER 1934 Rate of passage of inert materials through the digestive tract of the bovine. J. Dairy Sci., *17:* 297–305.

PAZUR, J. H., AND W. A. DELONG 1948 Effect of lignin content and stage of maturity of dry clover forage on the urinary excretion of aromatic acids by sheep. Sci. Agr., *28:* 39–46.

REID, J. T., P. G. WOOLFOLK, C. R. RICHARDS, R. W. KAUFMANN, J. K. LOOSLI, K. L. TURK, J. I. MILLER AND R. E. BLASER 1950 A new indicator method for the determination of digestibility and consumption of forages by ruminants. J. Dairy Sci., *33:* 60–71.

SKULMOWSKI, J., A. SZYMANSKI AND T. WYSZYNSKI 1943 Praktische Anwendung einer neuen, der sogennanten "qualitativen" oder Indikator — Methode zur Bestimmung der Verdaulichkeit des Futters. Ber. Landw. Forschunganst. des General-gouvernments, *1:* 76–104.

SNEDECOR, G. W. 1946 Statistical Methods Applied to Experiments in Agriculture and Biology. Iowa State College Press, Ames, Iowa.

SWIFT, R. W., E. J. THACKER, A. BLACK, J. W. BRATZLER AND W. H. JAMES 1947 Digestibility of rations for ruminants as affected by proportions of nutrients. J. Animal Sci., *6:* 432–444.

STUDIES OF DIETARY RESTRICTION AND REHABILITATION

II. INTERRELATIONSHIPS AMONG THE FAT, WATER CONTENT AND SPECIFIC GRAVITY OF THE TOTAL CARCASS OF THE ALBINO RAT [1]

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TWO FIGURES

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In studies of men (Behnke, Feen and Welham, '42; Welham and Behnke, '42) and of guinea pigs (Rathbun and Pace, '45) it has been shown that specific gravity varies inversely with the fat deposit and directly with muscle mass. Messinger and Steele ('49) found that for man the water content as well as the fat content can be estimated from specific gravity. However, Scheer et al. ('47) found a correlation of only — 0.0008 between the fat content and specific gravity of rats fed ad libitum on diets that contained different amounts of fat. A correlation of 0.121 for the same relationship was found in rats receiving limited amounts of the same diets. In view of these conflicting results, a further study on rats seemed desirable. Accordingly, as part of a study of changes occurring in male albino rats during dietary restriction and rehabilitation, the specific gravity of the animals and the correlations between it and the fat and water content of the total carcasses were determined.

[1] The opinions expressed in this paper are those of the authors and do not necessarily represent the official views of any government agency.

METHODS

Details concerning the methods employed and the composition of the diets in two separate studies of the 458 rats used in the present investigation have been published previously (Da Costa, Johnson and Berryman, '47; Da Costa and Clayton, '48, '50). The specific gravity of the shaved eviscerated rats (with tails removed) was determined by dividing their weights by volume displacements in water in a 2,000-ml cali-

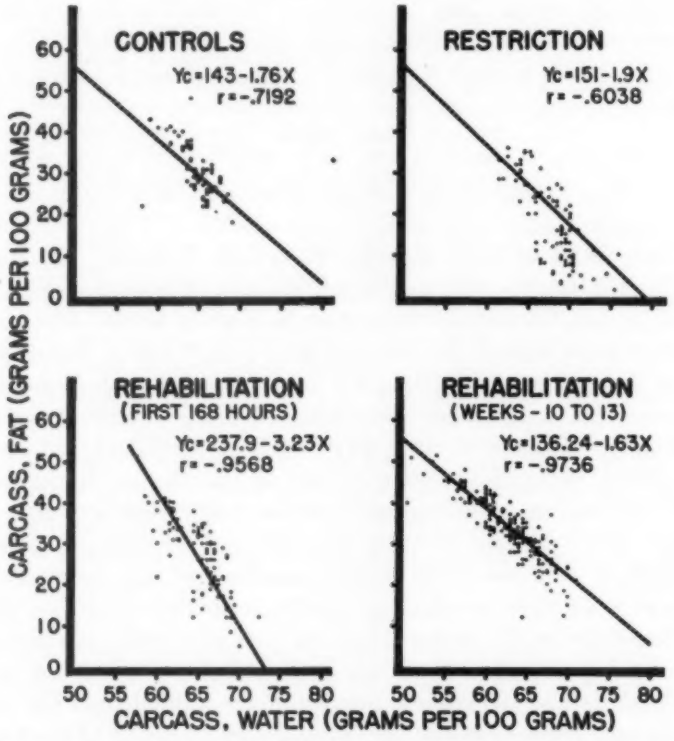

Fig. 1 Relation between total fat and water of whole carcass of male rats before, during and after dietary restriction. The regression lines are fitted to the data and the correlation coefficients are computed by the method of least squares.

brated cylinder. The eviscerated carcasses of the animals were ground until a macroscopically homogeneous mass was obtained. The water content of aliquots was determined by drying them to constant weight in a vacuum oven at about 110°C. The fat content was determined by the method of Eichelberger and Bibler ('40).

TABLE 1

Fat, water content and specific gravity of the whole carcass of male albino rats after dietary restriction and during rehabilitation

DIETARY REGIMEN		NUMBER OF RATS	DAYS ON DIETARY REGIMEN	FAT CONTENT	WATER CONTENT	SPECIFIC GRAVITY (Water 1.000)
				gm/ 100 gm	*gm/ 100 gm*	
		First study				
Control	Control	5	70	33.1	64.2	1.056
		2	77	32.0	63.5	1.058
		3	98	30.9	65.6	1.044
		4	140	28.2	65.8	1.059
Restriction	Low cal.[1]	8	70	13.5 [2]	71.6 [2]	1.087
	Carrot D.[1]	8	70	18.9 [2]	66.3 [2]	1.062
Rehabilitation	High prot.[1]	5	7	33.0	64.4	1.051
	after	5	28	27.4	67.1	1.055
	low cal.	5	70	30.6	65.4	1.056
	High fat	5	7	38.3 [2]	61.3	1.046
	after	5	28	36.9	60.5	1.044
	low cal.	5	70	37.7	61.6	1.067
	High CHO.[1]	5	7	32.4	63.6	1.057
	after	5	28	30.9	64.5	1.044
	low cal.	5	70	30.8	66.0	1.067
	High prot.[1]	5	7	32.0	63.6	1.049
	after	5	28	32.8	62.0	1.055
	carrot D.	5	70	33.6	63.7	1.053
	High fat	5	7	44.3 [2]	60.4	1.030
	after	5	28	41.5 [2]	59.2	1.039
	carrot D.	5	70	36.2	62.3	1.036
	High CHO.[1]	5	7	32.9	64.4	1.073
	after	5	28	39.1	61.1	1.058
	carrot D.	5	70	29.5	65.6	1.067
		Second study				
		A. Rehabilitation on a high protein diet				
Control	Control	6	98	29.5	63.5	1.047
		4	102	28.0	64.0	1.059
		4	140	28.9	64.1	1.058
		4	192	24.3	66.3	1.060

TABLE 1 — *continued*

DIETARY REGIMEN		NUMBER OF RATS	DAYS ON DIETARY REGIMEN	FAT CONTENT	WATER CONTENT	SPECIFIC GRAVITY (Water 1.000)
				gm/ 100 gm	*gm/ 100 gm*	
Restriction	Low cal.[1]	8	98	14.4 [2]	68.5	1.069
	Carrot D.[1]	8	98	27.5	64.9	1.059
	Syn. veg. sub.[1]	8	98	10.5 [2]	69.9	1.095
Rehabilitation	High prot.[1] after low cal.	2	1	9.0 [2,3]	68.8	1.081
		2	2	21.8	65.7 [2]	1.085
		2	3	28.8 [2]	65.5 [2]	1.075
		2	4	30.2 [2]	64.2 [2]	1.049
		8	42	33.5 [2]	62.5	1.044
		8	94	24.8 [2]	65.6	1.063
	High prot.[1] after carrot D.	2	1	27.5	68.6	1.067
		2	2	27.2	68.5	1.059
		2	3	17.8 [2]	68.1	1.091
		2	4	30.2	65.7	1.063
		8	42	30.0	65.2	1.068
		6	94	26.4	65.8	1.061
	High prot.[1] after syn. veg. sub.	2	1	15.2 [2]	68.8	1.099
		2	2	20.4 [2]	69.3	1.085
		2	3	23.6 [2]	67.7 [2]	1.086
		2	4	29.0	66.1 [2]	1.058
		8	42	32.0	63.0 [2]	1.055
		6	94	24.9	66.5 [2]	1.066
B. Rehabilitation on a high fat diet						
Control	Control	6	97	28.9	65.1	1.058
		4	101	28.9	65.1	1.058
		4	139	28.0	65.6	1.062
		4	176	24.2	66.3	1.072
Restriction	Low cal.[1]	8	97	14.4 [2]	69.1 [2]	1.089
	Carrot D.[1]	8	97	27.5	66.4	1.059
	Syn. veg. sub.[1]	8	97	9.7 [2]	69.9 [2]	1.084
Rehabilitation	High fat after low cal.[1]	2	1	16.1 [2]	67.2	1.060
		2	2	22.7	66.5	1.067
		2	3	32.2	61.8	1.046
		2	4	32.8 [2]	64.6 [2]	1.044
		7	42	40.0 [2,3]	58.3	1.020
		7	79	40.1 [2]	58.8	1.028
	High fat after carrot D.[1]	2	1	26.5	67.9	1.050
		2	2	28.5	64.5	1.057
		2	3	36.1	59.2 [2]	0.955
		2	4	33.9	61.1	1.045
		8	42	41.1 [2,3]	57.3	1.023
		7	79	42.0 [2,3]	57.9 [2]	1.036

TABLE 1 — *continued*

DIETARY REGIMEN		NUMBER OF EATS	DAYS ON DIETARY REGIMEN	FAT CONTENT	WATER CONTENT	SPECIFIC GRAVITY (Water 1.000)
				gm/ 100 gm	*gm/ 100 gm*	
Rehabilitation	High fat after syn. veg. sub.[1]	2	1	20.7	67.3	1.069
		2	2	21.5	67.8	1.065
		2	3	29.6	65.1	1.048
		2	4	37.0	60.7	1.038
		7	42	38.2 [2,3]	60.7	1.029
		8	79	36.0	62.1	1.041
	C. Rehabilitation on a high carbohydrate diet					
Control	Control	6	85	36.1	62.0	1.033
		4	89	35.0	62.8	1.035
		4	127	34.4	62.8	1.025
		4	196	34.3	62.7	1.037
Restriction	Low cal.[1]	8	85	9.81 [2]	70.3 [2]	1.080
	Carrot D.[1]	8	85	31.4 [2]	64.0 [2]	1.027
	Syn. veg. sub.[1]	8	85	17.9 [2]	67.8 [2]	1.060
Rehabilitation	High CHO. after low cal.[1]	2	1	6.7 [2]	70.0 [2]	1.068
		2	2	19.5 [2]	66.2 [2]	1.056
		2	3	12.9 [2]	66.7 [2]	1.060
		2	4	32.0	65.3 [2]	1.035
		8	42	31.9 [2]	64.1 [2]	1.038
		8	111	34.0 [2]	64.0 [2]	1.039
	High CHO. after carrot D.[1]	2	1	28.7	64.5	1.057
		2	2	26.6	63.5	1.040
		2	3	32.0	62.3	1.028
		2	4	30.5	66.1	1.038
		8	42	31.7	64.2	1.039
		7	111	34.1 [3]	64.0	1.049
	High CHO. after syn. veg. sub.[1]	2	1	10.2 [2]	69.1 [2]	1.078
		2	2	18.6 [2]	66.6 [2]	1.054
		2	3	16.7 [2]	66.3 [2]	1.055
		2	4	17.9 [2]	62.8	1.053
		8	42	33.0 [3]	63.1	1.049
		7	111	36.6 [3]	62.0	1.029

[1] Low cal., low calorie diet; carrot D., carrot diet; high prot., high protein; syn. veg. sub., synthetic vegetable substitute diet; high CHO., high carbohydrate diet.

[2] Values significantly different from those of controls (p is less than 0.05).

[3] Values significantly different from those in restriction (p is less than 0.05).

The correlation coefficients (r) between fat and water content, between specific gravity and fat, and between specific gravity and water, and the regression lines for these relationships, were obtained by using the method of least squares (Croxton and Cowden, '46).

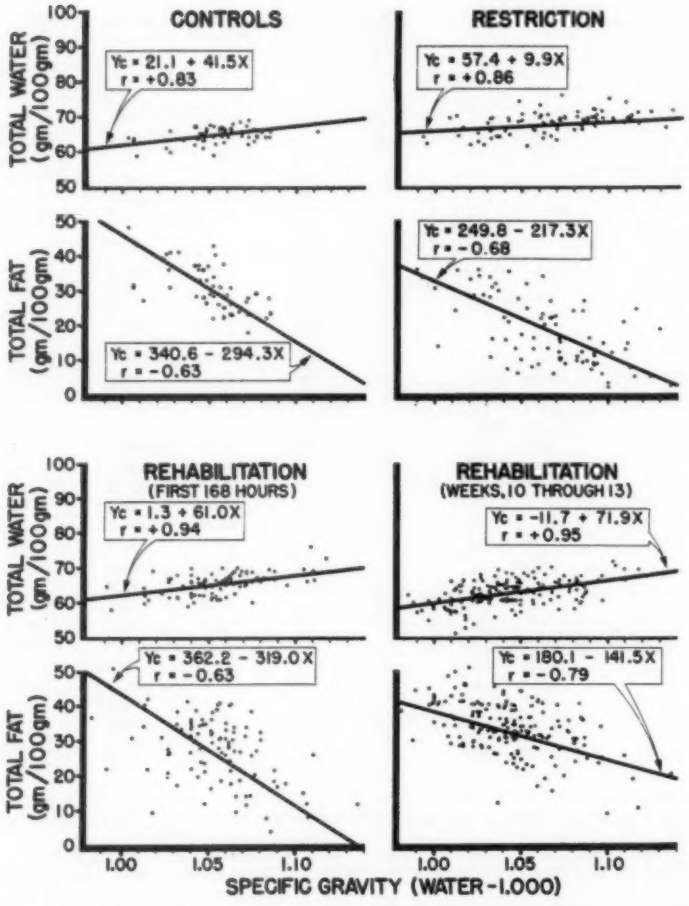

Fig. 2 Relation between specific gravity and either total carcass fat or water of male rats before, during and after dietary restriction. The regression lines are fitted to the data and the coefficients of correlation are computed by the method of least squares.

RESULTS

The data obtained in the first study on the fat and water content and the specific gravities of the rats on the different dietary regimens are presented in table 1. Inverse relationships between fat and water content are evident, regardless of the dietary regimen used. These are shown graphically in figure 1. Table 1 shows that the lowest fat content and the highest water content were found after 70 days of dietary restriction, and that the highest fat content and the lowest water content were found after rehabilitation on a high fat diet. The specific gravity varied inversely with the fat content and directly with the water content. In the second study (table 1) the same general relationships were found, although the periods of restriction and rehabilitation were longer and the rehabilitating diets relatively higher in protein, fat or carbohydrate.

The correlation coefficients and regression lines obtained by analyzing the combined data of the first and second study by the method of least squares are shown in figure 2. The direct relationship between water and specific gravity is indicated by the highly significant correlation coefficients of + 0.83 for control rats, + 0.86 for restricted rats, + 0.94 for rats after 7 days of rehabilitation, and + 0.95 for rats after 70 and 95 days of rehabilitation. The inverse relationship between fat and specific gravity is indicated by the correlation coefficients of — 0.63 for controls, — 0.68 for restricted, — 0.63 after 7 days of rehabilitation and — 0.79 after 70 and 95 days (fig. 2).

DISCUSSION

The inverse relationship between carcass fat and water supports the conclusions drawn by Haldi and Giddings ('42). Moreover, our findings of highly significant correlation coefficients between water content and specific gravity confirm the relationships reported by Messinger and Steele ('49) and Pace et al. ('47). The difference between our findings and those of Scheer et al. ('47) remains unexplained.

Our finding of an average specific gravity of 1.053 for the control rats agrees with Sarkisian's finding ('46) of 1.055 as the average specific gravity value for men physically acceptable for submarine duty. Also, our specific gravity values of 1.053 for normal control rats, and 1.079 after 70 days and 1.068 after 95 days of undernutrition, compare relatively well with the specific gravities found by Brozek ('46) of 1.071 for normal men, 1.084 after 12 weeks of semi-starvation and 1.089 after 24 weeks of semi-starvation.

Our data indicate that early in the rehabilitation period the specific gravity does not correlate as well with body fat and water content as in more stabilized nutritional states; namely, after a long period of restriction or after 70 to 95 days of dietary rehabilitation. The water and fat relationships evidently change rapidly during early rehabilitation. In view of the significant correlations between specific gravity and a wide range of fat and water content, the relationships found by us for the normal animal may likewise hold for chronic pathological conditions.

SUMMARY

The relations among fat and water content and the specific gravity of the total carcasses of 458 male albino rats were determined in connection with different types of dietary restriction and rehabilitation for varying lengths of time. The data were analyzed statistically for correlation coefficients (r) by the method of least squares.

The results show that an inverse relationship exists between carcass fat and water and between carcass fat and specific gravity, and that a direct relationship exists between carcass water and specific gravity. Also, the calculated regression lines may make the indirect determination of fat or water possible when specific gravity has been determined.

Specific gravity is, therefore, as good an index of the water content of the whole animal as it is of the fat content.

ACKNOWLEDGMENTS

Grateful acknowledgments are made to Dr. R. E. Johnson for his helpful suggestions and criticisms and to Major Floyd Votaw for his cooperation and assistance. Mr. Carl Gordon prepared the two charts.

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LITERATURE CITED

BEHNKE, A. R., B. F. FEEN AND W. C. WELHAM 1942 The specific gravity of healthy men. Body weight : volume as an index of obesity. J. Am. Med. Assn., *118:* 495.

BROZEK, J. 1946 Changes in specific gravity and body fat under conditions of experimental semi-starvation. Fed. Proc., *5:* 13.

CROXTON, F. E., AND D. J. COWDEN 1946 Applied Statistics. Prentice Hall, Inc., New York.

DA COSTA, E., AND R. M. CLAYTON 1948 Changes in the water content of the albino rat during the first week of dietary rehabilitation. Fed. Proc., *7:* 284.

———— 1950 Studies of dietary restriction and rehabilitation. I. Weight changes and food consumption in rats. J. Nutrition, *40:* 537.

DA COSTA, E., R. E. JOHNSON AND G. H. BERRYMAN 1947 Some effects of refeeding albino rats after caloric restriction. Fed. Proc., *6:* 405.

EICHELBERGER, L., AND W. BIBLER 1940 Water and electrolyte content of hydronephrotic kidneys. J. Biol. Chem., *132:* 645.

HALDI, J., AND W. GIDDINGS 1942 Dietary control of the water content of the skin of the albino rat. Am. J. Physiol., *135:* 392.

MESSINGER, W. J., AND J. M. STEELE 1949 Relationship of body specific gravity to body fat and water content. Proc. Soc. Exp. Biol. Med., *70:* 316.

PACE, N., L. KLINE, H. D. SCHACHMAN AND M. HARFENIST 1947 Studies in body composition. IV. Use of radioactive H_2 for measurement *in vivo* of total body water. J. Biol. Chem., *168:* 459.

RATHBUN, E., AND N. PACE 1945 The determination of total body fat by means of body specific gravity. Ibid., *158:* 667.

SARKISIAN, SARKIS S. 1946 The specific gravity of healthy men. Naval Med. Bull., *46:* 1207.

SCHEER, B. T., S DORST, J. F. CODIE AND D. SAULE 1947 Physical capacity of rats in relation to energy and fat content of the diet. Am. J. Physiol., *149:* 194.

WELHAM, W. C., AND A. R. BEHNKE 1942 The specific gravity of healthy men. Body weight : volume and other physical characteristics of exceptional athletes and of naval personnel. J. Am. Med. Assn., *118:* 498.

THE N BALANCE INDEX OF LOW-VALINE AMINO ACID MIXTURES AND OF WHOLE EGG PROTEIN IN THE ADULT RAT [1]

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THREE FIGURES

(Received for publication April 11, 1950)

A systematic investigation is being made in this laboratory of the biological value or nitrogen balance index of protein as related to its amino acid content. The present paper reports the results of experiments in which nitrogen balance indexes were determined for whole egg protein, an amino acid mixture simulating whole egg protein, and 4 amino acid mixtures low in valine.

METHODS

Two groups of adult male rats were used. Series 100 animals were 233 or 247 days old at the beginning and, after a 48-hour fast, weighed 208 to 282 gm (mean, 246 gm). They were fed 42.6 Cal./rat/day. Series 140 rats were 248 to 251 days old at the beginning and, after a 48-hour fast, weighed 280 to 338 gm (mean, 305 gm). They were fed 49.6 Cal./day/rat. Each experiment included the following sequence of diets: recovery diet (9.6% egg protein) two weeks, nitrogen free (N-free) diet one week, amino acid diet with half maintenance amount of nitrogen (half-N) one week, amino acid

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diet with full maintenance amount of nitrogen (full-N) one week. This 5-week cycle was repeated for each amino acid mixture or protein tested. Each group of rats was used in several such experiments, utilizing different amino acid mixtures. The series 100 rats were divided into two groups matched for body weight and one group was given the full-N diet before the half-N, which is the opposite of the usual order. This change in order of feeding made no significant difference in the results.

The egg protein used in experiment III of series 140 was prepared from fresh eggs by extraction with acetone and ether.

The "complete" amino acid mixture simulates whole egg protein in that it contains approximately the same amount of each naturally occurring essential amino acid per gram of total nitrogen. The non-essential amino acids of egg protein are replaced in this mixture by the D- forms of 6 essential amino acids plus sufficient L-glutamic acid to make the same total nitrogen.[3] The compositions of the complete amino acid mixture, the complete full-N diet, the N-free diet and the recovery diet are given in a previous paper (Anderson and Nasset, '48). A half-N diet is prepared by omitting half of the quantity of each amino acid and adding an equal weight of sucrose.

The one-sixth DL-valine amino acid mixture contains one-sixth as much DL-valine as the complete amino acid mixture. The missing valine is replaced by an isonitrogenous amount of L-glutamic acid. The slight change in physiological heat value brought about by this substitution is compensated for by adjustment of the sucrose content. The one-twelfth L-valine amino acid mixture contains one-twelfth as much valine as the total amount of DL-valine in the complete mixture. In each case the fraction refers to the proportion present of an amino acid, regardless of the enantiomorph concerned. Hence the one-twelfth L-valine mixture contains the same amount

[3] Amino acids and vitamins were supplied by Merck and Company. Folic acid was supplied by the Lederle Laboratories.

of L-valine per gram of total nitrogen as the one-sixth DL-valine mixture.

N-free, half-N and full-N diets were fed by stomach tube in two equal portions daily. Nitrogen intake was determined from analyses of the dietary components and of the whole diet. Feces were separated by Fe_2O_3 or Cr_2O_3 markers and preserved in sulfuric acid. Urine was collected for the last 4 days of each 7-day dietary period. Nitrogen determinations were made by the Kjeldahl method, using mercuric oxide as the catalyst. The other methods employed are described in previous papers (Anderson and Nasset, '48, '50).

RESULTS

The body weights given are averages of the weights at the end of the N-free, half-N and full-N periods. The animals gained 4% in the recovery period between experiments II and III of series 140 but otherwise they regained in the recovery periods only the small amount lost in the previous period of low nitrogen intake. Fecal nitrogen excretion was substantially constant within each experiment, which indicates that the digestibility of nitrogen was complete in all the diets. Amino acid mixtures or whole egg protein added to the N-free diet in "half maintenance" quantities resulted in a decrease in urinary nitrogen excretion in every experiment. Further additions, to "full maintenance" nitrogen intake, increased the urinary nitrogen on the low-valine diets; on the complete amino acid mixture or egg protein diets the urinary nitrogen was unchanged.

Series 100

Nitrogen balance data from series 100 rats are presented in table 1. Owing to gross inconsistencies of unknown cause, the data from experiment I, using the complete amino acid mixture, are omitted. This is not a serious loss because the performance on this mixture has been observed repeatedly and is usually rather uniform. Experiments II and III rep-

resent all of the remaining data in series 100; experiment III terminated this series because half of the animals were accidentally asphyxiated in an energy metabolism test.

In figure 1 nitrogen balance is plotted against nitrogen intake for series 100. Each point represents the average nitrogen balance status of the group of animals during the last 4 of 7 days on a diet. The slope of a line joining two points

TABLE 1

Average nitrogen balance data for rats receiving amino acid mixture diets

Series 100 rats		
Experiment number	II	III
Amino acid mixture	1/6 DL-valine	1/13 L-valine
Number of rats	11	7
Body weight (kg)	0.239	0.244
Metabolic body size ($kg^{3/4}$)	0.342	0.348
N balance data (mg N/day/$kg^{3/4}$)		
"N-free" period		
N intake	9	5
Fecal N	38	42
Urinary N	132	145
"Half-N" period		
N intake	79	85
Fecal N	34	35
Urinary N	114	129
"Full-N" period		
N intake	151	157
Fecal N	35	36
Urinary N	158	165

gives the fraction of nitrogen utilized of the increment in nitrogen intake from one diet to the other. This coefficient of nitrogen utilization has been given the symbol K′ and called the nitrogen balance index of ingested nitrogen (Allison et al., '46). It is equal to the product of K, the nitrogen balance index of absorbed nitrogen, and digestibility.

The three points in a single experiment do not lie on a straight line. The unusually large urinary nitrogen excretion on a N-free diet makes the corresponding point fall too low

on the graph. For this and other reasons (Anderson and Nasset, '50), the slope of the line joining the two uppermost points, K′ computed from the full-N and half-N diets, is preferred as an index of the quality of either a protein or an amino acid mixture. For easy comparison K′ computed from the full-N and N-free diets is also given in the figure.

In experiment II of series 100, K′ = 0.38. The poor utilization of ingested nitrogen indicated by this result demonstrates that reduction of the valine to one-sixth of the amount

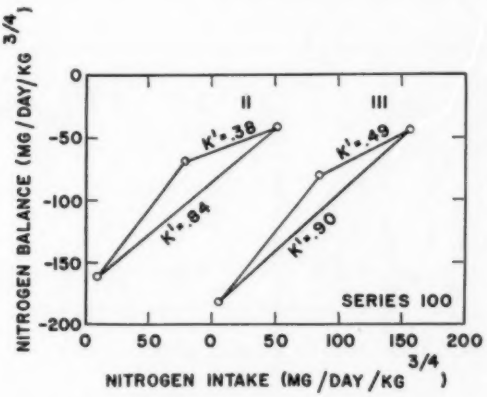

Fig. 1 Nitrogen balance index of ingested nitrogen, K′, for low-valine diets.
Experiment II — One-sixth DL-valine amino acid mixture.
Experiment III — One-thirteenth L-valine amino acid mixture.

in the complete amino acid mixture makes valine the limiting factor in this experiment. In an earlier paper it was shown that reduction of valine to one-third of the amount in the complete mixture is insufficient to affect utilization of the mixture (Anderson and Nasset, '48). In experiment III of series 100, one-thirteenth L-valine was used and K′ = 0.49. This mixture contained approximately the same amount of L-valine as the one-sixth DL-valine used in experiment II. The two values for K′ are not significantly different (P = 0.3). This result indicates that D-valine was not utilized.

TABLE 2

Average nitrogen balance data for rats receiving amino acid mixture diets

	Series 140 rats				
Experiment number	I	II	III	IV	V
Amino acid mixture	Complete	1/12 DL-valine	Egg protein	1/12 L-valine	Complete
Number of rats	13	8	8	8	8
Body weight (kg)	0.297	0.302	0.314	0.310	0.312
Metabolic body size ($kg^{¾}$)	0.403	0.407	0.420	0.416	0.418
	N balance data (mg N/day/$kg^{¾}$)				
"N-free" period					
N intake	4	4	4	4	4
Fecal N	46	40	34	34	37
Urinary N	135	160	138	146	122
"Half-N" period					
N intake	76	77	74	78	75
Fecal N	41	37	34	36	34
Urinary N	117	138	115	119	94
"Full-N" period					
N intake	150	147	143	149	146
Fecal N	42	38	37	39	34
Urinary N	122	177	100	152	93

Series 140

It was desired to extend the investigation of valine and the work was resumed later with the rats of series 140. The nitrogen balance data are given in table 2. No accidents intervened and these animals ran the full course of 25 weeks. Nitrogen balance is plotted against nitrogen intake in figure 2. Here again it is obvious that, with the possible exception

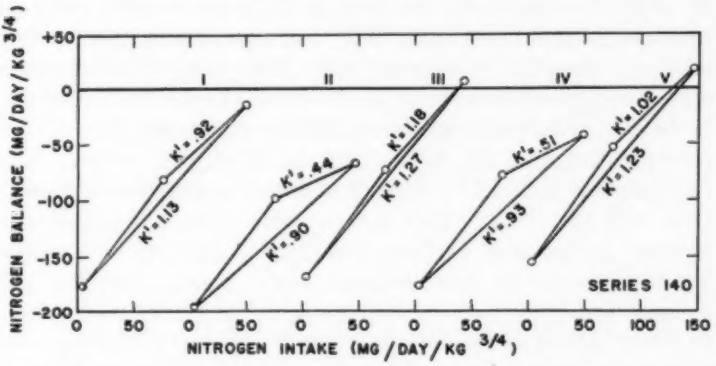

Fig. 2 Nitrogen balance index of ingested nitrogen, K', for low-valine and egg protein diets.

Experiment I — Complete amino acid mixture.
Experiment II — One-twelfth DL-valine amino acid mixture.
Experiment III — Egg protein as source of nitrogen.
Experiment IV — One-twelfth L-valine amino acid mixture.
Experiment V — Complete amino acid mixture.

of experiment III, the three points in a single experiment do not fall on a straight line.

The complete amino acid mixture was used in experiments I and V. $K' = 0.92$ in experiment I and 1.02 in experiment V. This difference is not significant ($P = 0.15$) but is in the direction to be expected after a considerable period on low protein diets. The computed nitrogen intake required to give equilibrium is less in experiment V (127 mg N/day/$kg^{3/4}$) than in experiment I (167 mg N/day/$kg^{3/4}$). This difference is highly significant ($P < 0.0001$) and is further evidence of

increased protein depletion with time on experiment (20 weeks from the end of experiment I to the end of experiment V).

In experiment III, using whole egg protein, $K' = 1.18$. This indicates a utilization of nitrogen from egg which is superior to that from either of the complete amino acid mixtures (experiments I and V).

The low-valine amino acid mixtures used in experiments II and IV were both significantly inferior to the complete amino acid mixture. $K' = 0.51$ for the one-twelth L-valine mixtures used in experiment IV. The one-twelth DL-valine amino acid mixture used in experiment II may be considered to be derived from the one-twelfth L-valine mixture by replacement of half the L-valine by the unnatural isomer. This replacement yields $K' = 0.44$, which is not significantly lower than 0.51 ($P = 0.3$). This comparison taken alone indicates at least partial and perhaps complete utilization of D-valine. Further consideration, however, casts some doubt on this assumption. The one-twelfth L-valine mixture used in experiment IV of series 140 gave results ($K' = 0.51$) very close to those obtained for the one-thirteenth L-valine used in experiment III of series 100 ($K' = 0.49$). These experiments were performed approximately a year apart and on different groups of animals. The results duplicate and confirm each other in every respect.

DISCUSSION

The failure of the one-twelfth DL-valine mixture (experiment II, series 140) to yield a K' significantly smaller than the one obtained for the one-twelfth L-valine mixture is difficult to explain. Perhaps a better notion of the meaning of these data is obtained by plotting nitrogen balance against the intake of valine nitogen, as is done in figure 3. Points on this plot that fall on the same abscissa may represent diets containing either the half or the full maintenance quantity of total N; the position on the abscissa is determined by the fraction of valine N in the total. If the points obtained from amino acid mixtures containing differing suboptimum amounts

of valine fall on a single curve, it is demonstrated that valine intake alone is the controlling factor in nitrogen retention. The exact intake of all the other amino acids, therefore, is not critical, provided of course that all of the diets contain a relative excess of each of the other essential amino acids and of total non-essential amino acids.

When plotted in this manner it is evident that the 4 mean values obtained from low DL-valine mixtures fall fairly well along a single curve. It is particularly significant that the two middle points are nearly coincident, because the point from series 100 was obtained with a half-N diet and the point

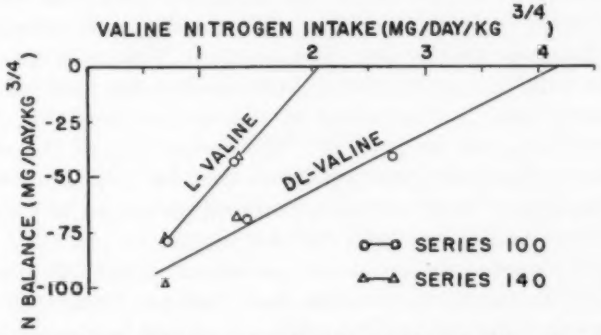

Fig. 3 Nitrogen balance plotted against intake of valine nitrogen.

from series 140 was obtained with a full-N diet. It is evident, therefore, that the position of these points is fixed by the intake of valine N and not by the total N of the diet. With the data from 38 individual determinations, the line of regression of nitrogen balance on DL-valine nitrogen intake was found to be:

$$NB = -109.2 + 26.0 \text{ (DL-valine nitrogen intake)}.$$

A line drawn through the mean values for the one-twelfth L-valine and one-thirteenth L-valine experiments is also shown in figure 3.

Extrapolation of a line on the diagram of figure 3 to the zero nitrogen balance value gives an estimate of the require-

ment of valine nitrogen to produce nitrogen equilibrium; i.e., the maintenance requirement for the adult rat. According to this method the mean maintenance requirement for all the animals receiving L-valine was 2.1 mg L-valine N/day/$kg^{3/4}$. The mean maintenance requirement for all animals (omitting one extreme value) receiving DL-valine was 4.2 mg DL-valine N/day/$kg^{3/4}$.

The reciprocal of the slope of a line on this diagram gives the fraction of a gram of nitrogen which must be supplied as valine in order to cause the retention of 1 gm of nitrogen by the rat. This fraction should be constant and reproducible for each essential amino acid, provided that the diet in which it is determined is deficient only in the single essential amino acid under investigation. This concept is in accord with the prevailing hypothesis that the utilization of all amino acids is determined by the amount available of the limiting essential amino acid. The reciprocals of the slopes of the two lines in figure 3 indicate that for nitrogen balance the fraction of total nitrogen required as valine nitrogen is 0.017 for L-valine and 0.038 for DL-valine.

The estimated requirement and the fraction of nitrogen required in the form of valine both indicate that D-valine is not appreciably utilized to replace L-valine in the adult rat receiving a low-valine diet.

It is realized that the extrapolations used in figure 3 may be too great for accurate predictions. Certainly extrapolations in the other direction to zero nitrogen intake do not correspond with the data presented in tables 1 and 2 for the N-free periods. All that can be said is that the valine requirement arrived at for the adult rat is the best one available at the moment. Further work should be done to furnish points on the curve nearer to the point of nitrogen equilibrium. It may very well be that a curvilinear relationship exists between nitrogen balance and valine nitrogen intake.

The whole egg protein used in experiment III is best compared with the complete amino acid mixture by using data for the 8 rats that participated in all experiments. On this

basis the individual values for experiments I and V were combined to give an average $K' = 0.99$ for both complete amino acid mixtures. This is to be compared wth the mean $K' = 1.18$ for the same rats on egg proten in experiment III. Since experiments I and V were equally spaced in time from experiment III, the effects of position in the series and progressive protein depletion are substantially eliminated. It is evident that under these conditions whole egg protein is significantly better utilized for the maintenance of nitrogen balance than the complete amino acid mixture ($P = 0.025$). This is accomplished despite the fact that the synthetic mixture is completely utilized (average $K' = 0.99$). In these experiments, then, the ingestion of the proteins of whole egg is actually accompanied by a retention of nitrogen which is greater than the amount ingested.

SUMMARY

Nitrogen balance was determined on adult rats receiving amino acid mixtures or whole egg protein as a source of nitrogen. Each experiment included a 7-day period on a N-free diet and a 7-day period on an amino acid mixture or an egg protein diet that supplied about half the maintenance requirement of nitrogen; this was followed by another 7-day period on a similar diet but with the nitrogen intake doubled. These diets were fed by stomach tube twice daily so that each rat received the same quantity of diet every day.

The nitrogen balance index of ingested nitrogen, K', for the complete amino acid mixtures is 0.99; for egg protein $K' = 1.18$. When valine is reduced to one-sixth, or less, of the amount present in the complete amino acid mixture, $K' = 0.51$ or less. Such a diminution of valine, therefore, reduces significantly the utilization of the nitrogen of the whole mixture.

A relationship is described between valine nitrogen intake and nitrogen balance. It is estimated that the adult rat, under these conditions, requires 2.1 mg of nitrogen from L-valine and 4.2 mg from DL-valine per day per kilogram$^{3/4}$. Using

the same relationship, it is estimated that the fraction of a gram of nitrogen that must be supplied as valine, in order to cause the rentention of 1 gm of nitrogen by the rat, is 0.017 for L-valine and 0.038 for DL-valine.

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LITERATURE CITED

ALLISON, J. B., R. D. SEELEY, J. H. BROWN AND J. A. ANDERSON 1946 The evaluation of proteins in hypoproteinemic dogs. J. Nutrition, *31:* 237.

ANDERSON, JOSEPH T., AND E. S. NASSET 1948 Nitrogen balance index and specific dynamic action in rats receiving amino acid mixtures low in isoleucine, methionine or valine. Ibid., *36:* 703.

———— 1950 The utilization by the adult rat of amino acid mixtures low in leucine. Ibid., *40:* 625.

STUDIES ON CAROTENOID METABOLISM

X. THE SITE OF CONVERSION OF CAROTENE TO VITAMIN A IN THE CHICK [1]

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There have been a number of recent reports which indicate that the site of the conversion of β-carotene to vitamin A in the rat is the intestinal wall. In an earlier study, Sexton, Mehl and Deuel ('46) noted that no vitamin A could be found in the livers of vitamin A-deficient rats which had been injected parenterally with carotene. Despite the relatively large amounts of β-carotene which had been deposited in the liver, the rats died of a vitamin A deficiency. It was suggested that the site of transformation of carotene to vitamin A might well be in the intestinal wall instead of in the liver, as had been formerly postulated. This would account for the fact that no carotene normally occurs in the blood or liver of rats after the oral administration of this carotenoid.

Mattson and collaborators ('47) were able to demonstrate after the feeding of carotene solutions by stomach tube that vitamin A appears in the intestinal wall before an appreciable amount is found in the liver. The quantity of vitamin A present in the intestinal wall was found to exceed that in the liver for a period of approximately 4 hours following the adminis-

[1] Aided by a grant from The Best Foods, Inc. The authors wish to express their appreciation for the use of the facilities of the Hancock Foundation. Contribution no. 257 from the Department of Biochemistry and Nutrition, University of Southern California.

tration of carotene. That the material formed in the intestinal wall actually is vitamin A was proved by Mattson ('48) since he was able to demonstrate that it had an absorption pattern similar to that of vitamin A and that it was adsorbed on a chromatographic column as a band fluorescent in ultraviolet light; finally, it yielded a single band chromatogram when it was mixed with known vitamin A and chromatographed.

More recent work has indicated that the intestinal conversion of carotene to vitamin A may not be confined exclusively to the rat. That such an extrahepatic change of carotene may likewise occur in certain other animals is indicated by the fact that the blood of a number of species is free of β-carotene. Although it is well known that the cow and man have considerable proportions of β-carotene in the blood serum, many other mammals such as the rabbit, pig, sheep and goat have no carotene in the blood. However, the possibility remained that carotene may be in the portal blood of such animals but that this carotenoid is removed quantitatively by the liver and therefore does not appear in systemic blood. This possibility has been answered in the negative by Goodwin, Dewar and Gregory ('46), who were unable to find any carotene in the portal blood of sheep and goats at intervals after carotene was introduced into the abomasum. When vitamin A was similarly administered, it was found in large amounts in the portal vein.

Goodwin and Gregory ('46) have extended the earlier experiments of Goodwin et al. ('46) by proving the absence of carotene in the portal blood of rabbits, goats and sheep after giving carotene by the oral, abomasal or duodenal routes; moreover, an increased vitamin A content was observed in the thoracic lymph after carotene was fed. Carotene was similarly found to be absent from the adrenal glands, liver, ovaries and bone marrow of goats.

The localization of the intestine as site of the transformation of β-carotene to vitamin A in the rat was also demonstrated by Glover et al. ('47, '48) simultaneously with Mattson et al. ('47). Thompson, Ganguly and Kon ('47, '49a, b)

have also reported independently a similar result on rats. Krause and Pierce ('48) have demonstrated by another procedure the extrahepatic conversion of carotene to vitamin A. The rate of increase of vitamin A in the plasma of normal rats and in rats having Eck fistulas was identical after the administration of carotene. If the liver were involved in the transformation of carotene to vitamin A, the appearance of the latter product should have been delayed in the Eck fistula animals.

Thompson, Ganguly and Kon ('49b) failed to obtain confirmation of the earlier results of Wagner ('39) indicating that carotene is changed to vitamin A in the intestine of the whale. Astaxanthin and preformed vitamin A were found in the intestinal contents of the Fin whales but no carotene was present. One possible explanation for the discrepancy between the results of Wagner and Thompson et al. is that the studies were not made under the same conditions. The whales examined by Wagner were obtained from the Arctic, while those studied by Thompson et al. came from the Antarctic.

The present experiments were designed to determine whether the chicken converts β-carotene to vitamin A in the intestinal wall or whether other organs may be involved in this reaction. The chicken differs from the rat, rabbit, goat and sheep in having considerable amounts of dihydroxycarotenoids in the liver, fat, ovaries and eggs (Ganguly and Deuel, '50). However, lutein, but no β-carotene, occurs in hen eggs or ovaries, in contrast to the ovaries of the cow, which contain both of these carotenoids.

METHODS

Day-old white Leghorn roosters [2] were placed on a synthetic vitamin A-low diet (table 1) on which a severe vitamin A deficiency has been shown to result within 21 to 28 days (Greenberg et al., '49). The chicks were housed in electrically heated brooders with raised screen floors. When vitamin A

[2] Obtained from the Pioneer Hatchery, Petaluma, California.

deficiency was produced, the chicks were ready for the tests described below. Since only limited numbers of birds could be used at one time, the remaining chicks were maintained until needed for testing on minimum amounts of β-carotene by giving daily doses of the carotenoid. This supplement was suspended for two days before the birds were to be used for the tests. Table 1 gives the composition of the diet.

The chickens were fasted for 12 hours before the tests were carried out. The birds were then given a dose of a

TABLE 1

The basal diet low in vitamin A used for depleting young roosters of vitamin A and carotenoids

CONSTITUENT	DIET 2
	%
Whole wheat (ground)	40
Barley (ground)	38
Commercial casein	12
Brewers' yeast (Anheuser-Busch, Strain G)	5
Cottonseed oil (Wesson) containing 2,400 chick units of vitamin D_3 per kilogram diet [1]	2
Iodized salt (0.02% KI)	1
Salt mixture [2]	2
Choline chloride [3]	

[1] Desynon (Winthrop), kindly furnished through the courtesy of Mr. C. Moser.

[2] The composition/100 gm: $CaCO_3$, 30 gm; KH_2PO_4, 30 gm; $CaHPO_4$ 25 gm; $MgSO_4 \cdot 7H_2O$, 9.9 gm; ferric citrate, 4.9 gm; $MnSO_4 \cdot 4H_2O$, 0.1 gm; $ZnCl_2$, 0.05 gm; $CuSO_4 \cdot 5H_2O$, 0.05 gm.

[3] Choline chloride was added to diet 2 (0.2 gm/kg).

cottonseed oil solution directly into the stomach by means of a stomach tube attached to a syringe. They were sacrificed at hourly intervals up to 6 hours, using anesthetization with chloroform. Control tests were made on roosters sacrificed after the 12-hour fast.

The liver was removed and the adhering blood was absorbed with a blotter. This organ was immediately weighed and heated with aqueous 50% KOH (1 ml/gm) plus 25 ml of 95% ethanol until completely dissolved. After cooling, water was

added to the solution to make a 50% alcoholic mixture. This was extracted with Skelly-solve A for three and one-half hours in a continuous liquid-liquid extractor. The extract was washed with water and dried with anhydrous sodium sulfate overnight.

The entire intestinal tract was removed except the cecum. The contents of the intestinal tract were flushed out by using 100 ml of a 0.9% sodium chloride solution, the tract was weighed, digested in KOH as described above, extracted with Skelly-solve and prepared for analysis in a manner similar to that used for the liver.

The carotene was determined from the E_{450} value on the Coleman Model 6A Junior Spectrophotometer. Vitamin A was estimated by the Carr-Price method. An aliquot of the Skelly-solve extract was evaporated to dryness in a stream of nitrogen, the residue was dissolved in 1 ml chloroform, and one drop of acetic anhydride was added to prevent subsequent cloudiness. Five milliliters of a saturated chloroform solution of $SbCl_3$ were added by the use of a calibrated syringe and the E_{620} value was recorded. β-carotene was calculated by the following formula:

$$\beta\text{-carotene (I.U./gram tissue)} = \frac{E_{620}}{0.231} \times \frac{\text{Dilution}}{0.6 \times \text{wt. of tissue}}.$$

The figure 0.231 represents the E_{450} value for 1 μg of β-carotene per milliliter. Vitamin A was estimated by the following formula:

$$\text{Vitamin A (I.U./gram tissue)} = \frac{E_{620} - (\text{micrograms carotene in sample} \times 0.00493)}{0.0199} \times \frac{\text{Dilution}}{\text{wt. of tissue}}.$$

Here 0.00493 is the E_{620} value for 1 μg of carotene and 0.0199 represents the E_{620} value for 1 I.U. of vitamin A ester.

Cottonseed oil containing 0.5% α-tocopherol was used as a solvent for carotene.[3] The solution contained 661 μg per milliliter. A vitamin A solution containing 12,580 I.U. per

[3] The carotene used was a preparation of General Biochemicals, Inc., which was 90% β- and 10% α-carotene.

milliliter in cottonseed oil having 0.5% α-tocopherol was used for the tests in which the length of time necessary to free the intestine from vitamin A was determined. Control tests involved feeding the cottonseed oil containing α-tocopherol without any provitamin A or vitamin A added.

TABLE 2

The vitamin A content of the livers and intestines of young chicks, previously depleted of vitamin A, at various intervals after the administration of a single dose containing 16,300 I.U. of vitamin A, the chicks being maintained on a vitamin A-low diet thereafter

ELAPSED PERIOD	NUMBER OF CHICKS	LIVER		INTESTINE	
		Average wet weight	Vitamin A/gm	Average wet weight	Vitamin A/gm
days		*gm*	*I.U.*	*gm*	*I.U.*
0	3	7.11	(0.26)[1]	11.75	(0.251)
1	2	8.99	131.0	11.83	5.053
2	2	8.80	167.6	12.86	0.397
3	2	8.03	162.4	....	....
4	2	7.40	15.9	11.09	0.417
5	2	8.04	25.7	12.96	0.178
6	2	7.53	127.4	11.62	0.519
7	2	9.23	100.9	11.94	0.319
11	2	8.90	94.3	12.89	(0.238)
13	2	11.69	7.8	14.08	(0.290)
17	2	9.03	7.9	13.88	(0.041)

[1] A brownish color developed with the Carr-Price reagent which gave a reading at 620 mμ, but no characteristic vitamin A response was noted.

RESULTS AND DISCUSSION

Table 2 records a series of tests on young vitamin A-depleted chicks [4] to determine the effect of the administration of vitamin A on the vitamin A content of the intestine and the liver. In these tests 6,300 I.U. of vitamin A (fish liver concentrate) were administered to each test chick. The chicks were sacrificed at various periods up to 17 days, during which they were maintained on the vitamin A-low diet. Table 3 gives a summary of the tests made also on vitamin A-depleted chicks after shorter periods of time (one to 6 hours), when they were

[4] See footnote 2, page 621.

given 0.5 ml of cottonseed oil alone (controls) or 0.5 ml of cottonseed oil containing 330 μg of carotene.

No detectable vitamin A was noted in the liver or intestinal wall of the chicks which had been maintained on the carotenoid-low-vitamin A-low basal diet. A brownish color developed with the Carr-Price reagent which gave a reading at 620 mμ,

TABLE 3

The vitamin A and carotene content of the livers and intestinal walls of vitamin A-depleted chicks after feeding cottonseed oil alone (controls) or at several periods after giving β-carotene dissolved in cottonseed oil by stomach tube

ELAPSED PERIOD	NUMBER OF CHICKS	AVERAGE WET WEIGHT		CAROTENE		VITAMIN A	
		Liver	Intestine	Liver	Intestine	Liver	Intestine
hours		*gm*	*gm*	*I.U.*	*I.U.*	*I.U.*	*I.U.*
Control animals							
1	2	7.57	19.34	(0.200)[1]	(0.274)	(0.114)	(0.098)
2	1	6.87	13.99	(0.650)	(0.371)	(0.271)	(0.191)
3	1	7.63	11.65	(0.831)	(0.665)	(0.308)	(0.100)
4	3	6.90	12.44	(0.751)	(0.535)	(0.200)	(0.172)
5	2	7.51	14.18	(0.563)	(0.750)	(0.185)	(0.201)
6	1	6.25	8.48	(0.368)	(0.347)	(0.153)	(0.806)
Test animals							
1	2	6.94	13.05	0.663	0.512	(0.715)	0.353
2	2	6.81	11.95	0.609	0.674	(0.554)	1.187
3	2	7.81	14.88	0.829	0.912	0.932	3.358
4	2	6.85	13.90	0.522	0.813	1.017	2.690
5	2	7.56	14.55	0.890	0.821	1.833	3.730
6	2	7.50	15.12	1.101	0.670	2.314	3.417

[1] With respect to the figures within parentheses: a brownish color developed with the Carr-Price reagent which gave a reading at 620 mμ, but no characteristic vitamin A response was noted; calculated and recorded as vitamin A.

but no characteristic vitamin A response was noted. Such abnormal reactions are calculated as vitamin A and recorded in table 3 by being enclosed in parentheses. After the administration of β-carotene, a prompt formation of vitamin A was noted in the intestinal wall; considerable quantities were found at both one and two hours after the carotene was fed, at which time no detectable vitamin A could be demonstrated in the liver. At three hours, vitamin A was first proved

to be in the liver. However, the proportion of the preformed vitamin A continued to be considerably higher in the intestine than in the liver for the periods up to 6 hours, at which time the tests were concluded. These results would seem to indicate that carotene is partly or wholly converted to vitamin A in the intestinal wall in the chick, just as is true of the rat, pig, sheep and goat.

The presence of vitamin A in the intestinal wall is not a reflection of its being transferred there from the liver. The vitamin A content of the wall is much higher two hours after the administration of carotene than after massive doses of vitamin A on any but the first day. Thus, in spite of the liver vitamin A's being as high as 168 I.U/gram in the vitamin A-fed rats, that present in the intestinal wall remained at a very low concentration. The liver vitamin A levels obtained in such instances were almost 75 times the maximum reached 6 hours after feeding carotene. It would thus appear that the intestinal vitamin A present after carotene ingestion does not originate in the liver, to be then carried back to the intestinal wall.

The presence of carotene in the intestinal wall extracts may be explained on the basis that the pigment had been absorbed and was in the process of being converted to vitamin A in that location. Another suggestion is that carotene had not actually been absorbed, but that the pigment present in the extracts might be traced to carotene adhering to the mucosa which had not been removed by washing and was mixed with the tissue when it was washed. However, the chromogenic material in the liver after carotene feeding had some of the characteristics of carotene; if this carotenoid is actually β-carotene, one might be forced to assume that the intestine is not the sole site for the degradation of β-carotene in the chick, but that some breakdown occurs at other sites.

However, two additional possible explanations occur to us. In the first place, it is altogether possible that the chromogen may be a dihydroxycarotenoid such as lutein, since this compound is known to be present in liver and to originate

from dietary sources (Ganguly and Deuel, '50). A still more plausible explanation is that the material may be partly or wholly α-carotene. In fact, a spectrophotometric examination of the pigment after purification by chromatography showed it to have the characteristics of α-carotene. Recent unpublished experiments in this laboratory (Ganguly and Deuel, '50) have indicated that while pure β-carotene cannot be absorbed by the chick, α-carotene is readily absorbable. The carotene preparation used contained about 10% of the α-isomer.

SUMMARY

When β-carotene was fed to vitamin A-depleted chicks, vitamin A first appeared in the intestinal wall after one hour and in the liver after three hours.

The proportion of vitamin A per gram of tissue was constantly higher in the intestinal wall than in the liver for a 6-hour period after the feeding of carotene.

When massive doses of vitamin A were fed to chicks, the amount of vitamin A in the intestinal wall fell to a relatively insignificant level on the second day in spite of the fact that the concentration of vitamin A in the liver was extremely high. It is believed that this fact means that the presence of large concentrations of vitamin A in the liver does not cause a concomitantly high concentration in the intestinal wall.

These data are interpreted as proof that the chick is able to change β-carotene to vitamin A in the intestinal wall.

LITERATURE CITED

GANGULY, J., AND H. J. DEUEL, JR. 1950 Unpublished observations.

GLOVER, J., T. W. GOODWIN AND R. A. MORTON 1947 Conversion of β-carotene into vitamin A in the intestine of the rat. Biochem. J., *41:* xlv.

———— 1948 Studies in vitamin A. 8. Conversion of β-carotene into vitamin A in the intestine of the rat. Ibid., *43:* 512.

GOODWIN, T. W., A. D. DEWAR AND R. A. GREGORY 1946 The transport of absorbed carotene in herbivora. Ibid., *40:* lx.

GOODWIN, T. W., AND R. A. GREGORY 1948 Studies in vitamin A. 7. Carotene metabolism in herbivores. Ibid., *43:* 505.

GREENBERG, S. M., C. E. CALBERT, J. H. PINCKARD, H. J. DEUEL, JR. AND L. ZECHMEISTER 1949 Stereochemical configuration and provitamin A activity. IX. A comparison of all *trans*-γ-carotene and pro-γ-carotene with all-*trans*-β-carotene in the chick. Arch. Biochem., *24:* 31.

KRAUSE, R. F., AND H. B. PIERCE 1948 The extrahepatic conversion of carotene to vitamin A. Ibid., *19:* 145.

MATTSON, F. H. 1948 The site of conversion of carotene to vitamin A. J. Biol. Chem., *176:* 1467.

MATTSON, F. H., J. W. MEHL AND H. J. DEUEL, JR. 1947 Studies on carotenoid metabolism. VII. The site of conversion of carotene to vitamin A in the rat. Arch. Biochem., *15:* 65.

SEXTON, E. L., J. W. MEHL AND H. J. DEUEL, JR. 1946 Studies on carotenoid metabolism. VI. The relative provitamin A activity of carotene when introduced orally and parenterally in the rat. J. Nutrition, *31:* 299.

THOMPSON, S. Y., J. GANGULY AND S. K. KON 1947 The intestine as a possible site of conversion of carotene to vitamin A in the rat and pig. Brit. J. Nutrition, *1:* v.

———— 1949a The intestinal conversion of carotene to vitamin A. Biochem. J., *44:* ix.

———— 1949b The conversion of β-carotene to vitamin A in the intestine. Brit. J. Nutrition, *3:* 50.

WAGNER, K. H. 1939 Vitamin A and β-Carotin des Fin-, Blau-, and Spermwals. Johann Ambrosius, Barth, Leipzig.

THE USE OF CHROMIC OXIDE AS AN INDEX FOR DETERMINING THE DIGESTIBILITY OF A DIET [1]

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TWO FIGURES

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INTRODUCTION

Digestibility trials have provided a basis for acquiring much of our present knowledge of the nutritive value of the components of both human and animal diets. Such trials are still of fundamental importance in nutrition research. One of the chief problems in this type of work has been to identify the fecal output resulting from a specific intake of feed.

To solve this difficulty, most digestion work has involved "time collection" of feces. In the case of humans and non-ruminants, markers have frequently been included in the ration. The possibilities of errors arising from such practices are numerous; and in addition this procedure involves the keeping of records of daily feed intake, as well as a daily quantitative collection of all feces output.

A number of workers have explored the possibility of including a known amount of a completely indigestible material in the feed and, by determining its subsequent concentration in the feces, allowing this material to serve as an index of digestibility of that feed. From such data the digestion co-

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efficients for the different nutrients of a food can be calculated according to the following formula:

$$\text{Digestion coefficient} = 100\ \left(\frac{a-b}{a}\right),$$

where a = parts of nutrient per unit of index substance in the food, and b = parts of nutrient per unit of index substance in the feces.

The use of chromic oxide (Cr_2O_3) as such an index substance was first proposed by Edin ('18).

Hamilton, Mitchell, Kick and Carman ('27–'28) found in experiments with sheep a good agreement between the conventional (quantitative) fecal collection and the Cr_2O_3 method, provided collection periods were of three days or longer in duration. More recently, satisfactory results with Cr_2O_3 as an indicator for digestibility measurements have been reported by Andersen and Frederiksen ('35); by Skulmowski, Szymanski and Wyszynski ('43) with sheep and horses; by Barnicoat ('45) with sheep, calves and pigs; by Olsson, Kihlen and Cagell ('49) with horses; and by Kane, Jacobson and Moore ('49) with cows. Virtanen ('50) has also reported the successful use of Cr_2O_3 with humans. All these experiments indicate that the Cr_2O_3 method can replace the conventional procedures for determining digestibility of rations or nutrients consumed.

As a main advantage, the method leads to a simplified experimental procedure by avoiding the necessity of a quantitative record of either food intake or feces output. The chemical work, however, is increased by the necessary determination of the Cr_2O_3 content of food and feces. An easy, rapid, and acceptably accurate analytical method for Cr_2O_3 is therefore of primary importance to the usefulness of this material as an index substance.

The chemical determination of chromic oxide

Methods for determining Cr_2O_3 have been proposed by Paloheimo and Paloheimo ('35), by Edin, Kihlen and Nordfeldt ('44), and by Barnicoat ('45).

Studies in this laboratory have resulted in the colorimetrical determination described below, which is a modification of the methods of Paloheimo and Paloheimo, and Barnicoat.

PROCEDURE

One to 2 gm of feed or feces, containing from 20 to 50 mg Cr_2O_3, are ashed in a 75-ml nickel crucible at about 600°C. After cooling, approximately 1 gm of Na_2O_2 is added, mixed well with the ash by swirling the crucible, and the mixture fused at gentle heat until liquid. Continue heating for about 5 minutes at low red heat, swirling the crucible occasionally. When cold, place the crucible in a 500-ml beaker and add cold distilled water to dissolve the residue. Let stand 5 to 10 minutes, then transfer the solution into the beaker. Wash the crucible thoroughly with hot distilled water. Leave the solution standing in the beaker for about 30 minutes, then filter into an Erlenmeyer flask and wash the residue with warm distilled water. Transfer the filtrate into a 500-ml volumetric flask and make up to volume with distilled water. Measure light transmission with a photoelectric colorimeter, using a 440 mμ filter, and with distilled water as a blank. Determine the amount of Cr_2O_3 from a calibration curve obtained with the same cuvette.

Determination of calibration curve from standard solutions

The calibration curve shown in figure 1 was derived from 34 determinations of transmittance with known amounts of chromé-green (Cr_2O_3) including:

(a) Samples of Cr_2O_3 from different origins, fused directly or heated in a muffle oven at ashing temperatures before fusion; (b) samples of sheep feces to which Cr_2O_3 was added; (c) secondary dilutions of original solutions.

The good agreement of these differently obtained points with the standard indicates the general applicability of the method.

The extinction values corresponding to the observed per cent transmittance (photometric density = 2-log transmittance) showed no linear relationship to the concentration of Cr_2O_3, indicating that the Beer-Lambert law is not applicable in this case. Plotting y against $1000 \cdot \frac{1}{x}$ (fig. 2) gave a straight line, proving that the data of figure 1 were correctly represented by the general equation $y = \frac{a}{x} + b$, where y is the concentration of Cr_2O_3 and x the per cent transmittance of the solution.

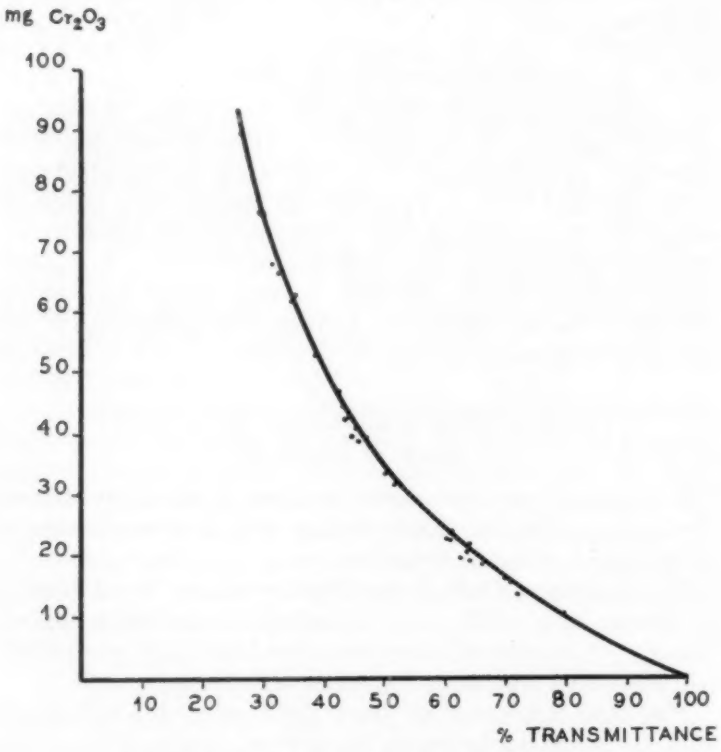

Fig. 1 Calibration of standard Cr_2O_3 solutions.

The determination of digestibility

The successful use of chromic oxide as an index of the digestibility of a diet demands that random samples only of feces need be retained for analysis of the concentration of the index. To obtain data relative to the usefulness of the analytical procedure proposed a test was conducted, using rats, in which the digestibility of the dry matter eaten was calculated both by the conventional time collection method and by the index method. The colony stock diet was finely

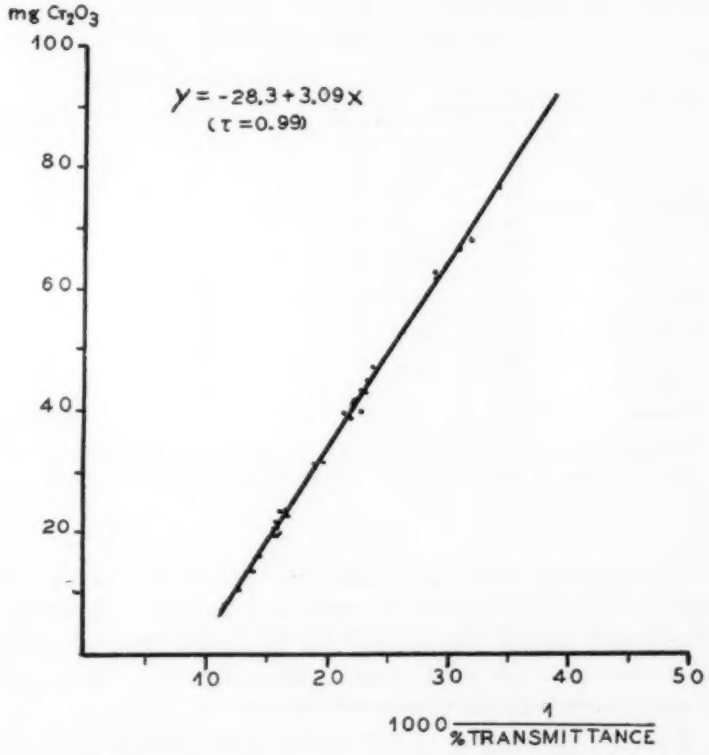

Fig. 2 Regression of Cr_2O_3 concentration on 1,000 times the reciprocal of the per cent transmittance.

ground and chrome-green added so that the mixture contained 1% chromic oxide (Cr_2O_3). This diet was fed for a 5-day preliminary period, followed by a 6-day collection period. Feces collections were made in the morning, at noon, and in the evening. The 6 morning collections were combined for each animal, as were the noon and evening collections. The three composite feces samples from each rat, as well as appropriate feed samples, were analyzed for their chromic oxide content by the method described above.

TABLE 1

Dry matter digestion coefficients[1]

RAT NO.	CONVENTIONAL TIME COLLECTION METHOD	CHROMIC OXIDE INDEX METHOD		
		Morning collection	Noon collection	Evening collection
	%	%	%	%
1	74	73	72	72
2	72	70	70	72
3	66	65	64	65
4	70	71	69	69
5	72	73	72	71
6	74	74	73	73
7	72	74	71	70
8	71	70	71	70
9	66	62	66	63
10	74	71	72	71
Averages	71	70	70	70

[1] Standard deviation of single coefficients = ± 1.86 units of per cent.
Standard error of means of 10 rats = ± 0.6 units of per cent.

RESULTS

The apparent digestion coefficients of the dry matter of the diet calculated by the conventional method, using total intake and output figures, and by the index method using separately the morning, noon, and night collections, are shown for each of the 10 rats in table 1.

Analysis of variance of the digestion coefficients (table 2) indicated that only the differences between individual rats were statistically significant; and that only once in 20 cases

would the coefficient determined by the time method as compared to that by the index method differ by more than 5.1 percentage units in the case of a single rat, or by more than 1.5 percentage units in the case of the averages of 10 rats.

It seems evident that the different samples of the day's feces output by the rats contained the same concentrations of the chromium. Thus, any random samples of the feces could be used equally well for determining the digestibility. It is also evident that the chromium analysis procedure is satisfactory, since the digestion coefficients obtained by its use were not different from those determined by the conventional time collection method.

TABLE 2

Analysis of variance of dry matter digestion coefficients

SOURCE OF VARIATION	DEGREES OF FREEDOM	VARIANCE s^2	VARIANCE RATIO	
			Observed	Necessary minimum (P = 0.05)
All causes	39	11.95		
Analyses	3	3.80	1.07	2.96
Conventional vs. Cr_2O_3	1	10.21	2.87	4.21
24-hour intervals	2	0.60	0.17	19.45
Animals	9	39.86	11.22	2.24
Remainder (between coefficients for same rat)	27	3.55		

LITERATURE CITED

ANDERSEN, A. C., AND L. FREDERIKSEN 1935 Verdaulichkeitsversuche an Milchkühen. Biedermanns Zentralbl. f. Agriculturchemie u. rationellen Landwirtschaftsbetr., *5:* 334.

BARNICOAT, C. R. 1945 Estimation of apparent digestibility coefficients by means of an inert "reference-substance." N.Z.J. Sci. Technol., *27:* 202–212.

EDIN, H. 1918 Orienterande försök over en på ledkroppsprincipen grundad metod att bestämma en foderblandnings smältbarhet. Medd. Nr. 105 från Centralanst. for försöksväsendet på jordbrüksomradet.

EDIN, H., G. KIHLEN AND S. NORDFELDT 1944 A summarized description of "Edin's indicator method" for the determination of the digestibility of feeds and feed mixtures. Lantbrukshögskolans Annaler, *12:* 166–171.

HAMILTON, T. S., H. H. MITCHELL, C. H. KICK AND G. G. CARMAN 1927–28 New method tested in digestion trials. 41st Ann. Rep., Ill. Agr. Expt. Sta., pp. 119–121.

KANE, E. A., W. C. JACOBSON AND L. A. MOORE 1949 A comparison of techniques used in conducting digestibility studies on dairy cattle. 41st Ann. Meeting, Am. Soc. of Animal Production.

OLSSON, N., G. KIHLEN AND W. CAGELL 1949 Smältbarhetsförsök med hästar. Landbrukshögskolan Hüsdjürsförsökanstalten. Medd., 36.

PALOHEIMO, L., AND I. PALOHEIMO 1935 Eine photometrische Methode zur Bestimmung des Cr_2O_3 bei Verdaulichkeitsuntersuchungen nach sog. quantitativen Indikatorverfahren. Tierernährung, *7:* 317–324.

SKULMOWSKI, J., A. SZYMANSKI AND T. WYSZINSKI 1943 Praktische Anwendung einer neuen, der sogenannten "qualitativen" oder Indikator-Methode zur Bestimmung der Verdaulichkeit des Futters. Landw. Forschungsanst. des Generalgouvernements.

VIRTANEN, A. I. 1950 Correspondence to the authors.

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